ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTES OF HEALTH

1960

NATIONAL CANCER INSTITUTE
VOLUME II

NATIONAL INSTITUTES OF HEALTH
PUBLIC HEALTH SERVICE
U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

" Report of program activities.

NONCLINICAL PROGRAMS

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NONCLINICAL PROGRAMS

Members of the staff who participate in the nonclinical programs continue to create new knowledge that enhances our understanding of cancer, entails development of new concepts, and provides a reservoir of laboratory findings important for future application to the problems of cancer and other diseases. The joint presence of this group of research workers and the clinical investigators within one sizable Institute provides the means for an exchange highly beneficial to both groups. The function of the nonclinical intramural program may be viewed as the creation of new knowledge that is well ahead of that required for application to problems of man, and the yields of the program form a wellspring which must be maintained with a vigor that insures a wholesome level. Among the most important ingredients is the continual supply of new concepts since they are the key to bringing new approaches to bear on the cancer problem beyond those receiving current consideration. Another important ingredient is the development of concepts already at hand to a stage sufficient for adequate evaluation. Collaboration by investigators in different disciplines, founded upon mutual interest, understanding, and respect, can do much to bring about new concepts and to speed along their development. In a number of cases, sizeable groups, ably led, provide greatest progress. In other cases, the mature investigator working independently makes his greatest contribution. Oftentimes the investigators who create new concepts and make initial development of them can make their best contribution when they do not carry through the application of the findings, but rather are left free to develop new concepts, understanding and evaluation from the purely scientific standpoint. In other cases, the investigator who initiates a study may effectively carry it through to a point where its applicability to an evident problem is attained.

The summarization which follows of the research progress of the nonclinical intramural staff attests the continuing highly important contributions made by the Institute staff. While all areas of cancer research are not fully represented, the staff is producing research of a very high order in many areas believed to be of significance for the future development of cancer research.

STUDIES ON THE INDUCTION OF CANCER

Several investigators throughout the Institute continue to make contributions toward our understanding of the factors underlying the causation of cancer. Many aspects of this problem warrant increased effort, both in terms of the materials in the environment to which man is exposed, singly or in combination, and the mechanisms within the living organism that are involved in initiation of the cancer process.

Chemicals:

Studies progress on the organic fractions of the particulate phase of urban air pollutants, as part of a comprehensive and prolonged program dealing with the relationships between air pollutants and health hazards, by Drs. W. C. Hueper and W. W. Payne in collaboration with Drs. P. Kotin and H. Falk (University of Southern California) and Messrs. E. Tabor and E. Sawicki (Taft Sanitary Engineering Center). Experiments with mice receiving the powerful carcinogen, benzo[a]pyrene, which serve as a reference standard for comparing relative potency of the solvent fractions of air pollutants, confirmed the concept that a low-level recurring exposure to a carcinogen is more hazardous than a single exposure to the same amount. In addition to confirming the presence of benzo[a]pyrene in samples collected by filtration of air from 8 large U. S. cities, the group has also shown carcinogenicity of samples not containing benzo[a]pyrene. Distinct variations in composition of air pollutants from different cities were demonstrated in terms of biological and chemical parameters. The percentage of tumors observed differed with the chemical fraction studied as well as the city from which the sample was obtained. No consistent correlation between the degree of carcinogenic potency, the amount of benzo[a]pyrene per sample, and the lung cancer mortality rate reported for a given city was found.

Preliminary studies on testing eluates of carbon adsorbates from raw river waters for carcinogenicity have produced several cancers in mice receiving samples subcutaneously.

Drs. Hueper and Payne have extended the studies on carcinogenicity of chromium-containing materials. Chromite ore roast, which contains various compounds of chromium with different solubilities, produces tumors in rats, and the list of chromium compounds shown to be carcinogenic has been extended, with the addition now of a trivalent chromium compound along with other hexavalent species. Moderately soluble (water) hexavalent chromium compounds display the highest carcinogenicity.

Prolonged inhalation by rats of dusts of chromic oxide and the chromates of barium, lead and zinc (compounds widely used as pigments and hence in contact with many workers via the respiratory route,) has produced green deposits, adenomatous changes and squamous cell metaplasia in the lungs of the exposed animals, but no primary cancers.

Because tumor-inducing effects of certain-water insoluble polymers have been interpreted by some investigators as resulting from physical factors (e.g., surface forces) rather than chemical ones, Dr. Hueper investigated the tumor-producing abilities of chemically different polymers of this type implanted subcutaneously or intraperitoneally in rats and mice in different physical forms (cube, sheet, disk, film foam, and powder). Although data obtained with polyethylene are consistent with the interpretation that physical factors play the key role in carcinogenesis with polymers, the data obtained with polyurethane are against the concept.

Because previous testing of polyvinylpyrollidones (PVP) containing a wide range of molecular weights gave inconsistent results as to their carcinogenic properties and the variability of the findings might have been due to differences in retention of some of the larger polymer species, Dr. Hueper re-evaluated the carcinogenic properties of PVP preparations containing either small (average molecular weight of about 10,000) or large (average molecular weight of about 50,000) molecular species. Although deposits of homogeneously staining material were seen in various tissues in the rats and rabbits injected with PVP of both large and small molecular species, the cancers that appeared were the same as those seen in the controls (frequency, location and morphological types). The smaller molecular weight PVP molecules were retained by the glomerular filter in the kidneys of the rat, but not in those of the rabbit.

Several investigators have produced cancers of the urinary bladder in mice by surgically implanting pellets of paraffin wax or cholesterol containing a carcinogen into the bladder by a method developed by Jull. However, bladder tumors have also appeared in the control animals with pellets of paraffin wax or cholesterol alone. Drs. W. Conway, W. Hueper and R. Feldstein and Miss E. Lethco have, therefore, prepared pellets made of synthetic wax (hexadecyl palmitate) and have incorporated into the pellets in addition to the dyes under test, varying amounts of powdered cellulose to provide greater permeability of fluids into the pellets. They have shown that more rapid release of the test substance from the pellet occurs with higher proportions of cellulose, thus providing the means for controlled release of the potential carcinogen under study. With this new technique for evaluating substances for carcinogenicity, several azo dyes are under study.

Dr. Conway and Miss Lethco are also investigating the metabolism of several azo dyes by other techniques. Utilizing sensitive colorimetric and radioactive tracer techn iques, they have observed several colored and colorless metabolic products in the urine. The percentage of the dose which appears as urinary metabolites increases as the dose decreases.

Drs. M. G. Kelly and R. W. O'Gara have added to their observations on chemical carcinogenesis in newborn mice. After implantation of either 0.02 mg. dibenz(a, b)anthracene (DBA) or 0.011 mg. 3-methylcholanthrene (MC) into noninbred albino mice during the first 18 hours after birth, 100 percent of the animals developed pulmonary tumors by 16-29 weeks. Subcutaneous fibrosarcomas arising from perimysium appeared at the site of injection of either carcinogen. Mice given 3-methylcholanthrene developed fulminating leukemia 9-16 weeks after injection. In collaboration with Mr. Nathan Mantel, the dose response relations were studied. The smallest single doses producing tumors are: Fibrosarcomas -- 0.0002 mg. DBA and 0.0004 mg. MC; lung tumors -- 0.0067 mg. DBA and 0.0037 mg. MC. At high doses of MC, 30 percent of albino mice developed leukemia and 70 percent of C3H male mice developed hepatomas. The production of tumors with such small doses suggests the value of the newborn animal as a test subject for potential carcinogens of greater sensitivity than that available formerly.

In studies aimed at inducing lung cancers, Dr. M. Stanton and Messrs. R. Blackwell and J. Albrecht have produced metastasizing squamous-cell carcinomas of the lung in rats by intravenous injection of a suspension of methylcholanthrene in tricaprylin carried to the lung in an halogenated aliphatic hydrocarbon (hexachlorotetrafluorobutane). The tumors apparently arose in areas of bronchial metaplasia, probably arising from halocarbon-produced infarction. Ultraviolet light microscopy indicated deposition of methylcholanthrene at the tumor site.

Dr. E. W. Chu, with Dr. K. M. Herrold, is engaged in a study of the effects of carcinogens on the female genital tract of Syrian hamsters. Hamster cervices painted with 9,10-dimethyl: 1,2-benzanthracene showed atypical cytological changes in the vaginal smears after 1-1/2 months, and definite carcinomatous changes in 2 months. 3,4-Benzpyrene-painted cervices showed comparable cytohistological findings after a much more prolonged period. Cytological changes have been noted in vaginal smears obtained from animals whose cervices were painted with tobacco tar dissolved in acetone only after 10 months.

Of 400 hamsters that received in the cheek pouch pellets containing suspected carcinogens, Dr. L. Dunham found that 24 animals displayed

lesions in the pouch, of which 19 were inflammatory in nature. One squamous papilloma was seen and four examples of epithelial hyperplasia were noted.

Further work on the hemangioendotheliomas of liver and spleen that developed following intravenously injected thorotrast (colloidal thorium dioxide) has been performed by Dr. R. Swarm. Metastases to spleen and liver occur following subcutaneous transplantation. Dr. P. Mori-Chavez has found visceral metastases with these tumors more frequent at high altitudes (in Peru) than at sea level. Dr. Swarm has also reviewed in several European laboratories tissue specimens from human patients who were given thorotrast.

Dr. M. Potter is investigating mouse strain relationships and the induction of leukemia by skin painting with methylcholanthrene. Approximately 80 percent of one-month-old $DBA^f/_2$ mice painted with the carcinogen developed generalized leukemia. No leukemias appeared in strain BALB/c mice which had been painted in a similar way. The F_1 hybrids of BALB/c and DBA/2 also did not develop leukemia after skin painting. The animals of the first generation backcross to $DBA^f/_2$, however, had an incidence of about 25 percent leukemia. Studies are being extended to a family study of second generation backcross mice.

Mechanisms of Carcinogenesis:

Dr. H. Sidransky is investigating a number of factors influencing the pathogenesis of rat hepatomas induced by certain chemical carcinogens. Although liver regeneration in partially hepatectomized rats is somewhat inhibited in rats ingesting ethionine in comparison with rats on the control diet, the histologic changes in the liver of the experimental animals are similar to those seen in nonhepatectomized animals ingesting ethionine. However, rats with partial hepatectomies and ingesting ethionine appear to have a delayed induction period for hepatomas. p-Hydroxypropiophenone, which inhibits the development of liver tumors induced by butter yellow, was found to inhibit ethionine-induced hepatomas. With Dr. H. Morris, Dr. Sidransky found that male rats show a higher incidence of liver tumors when fed N-2-fluorenylacetamide than female rats. If the survival of female rats is prolonged by removing breast tumors, the incidence of hepatomas can be increased appreciably in the female animals.

Dr. Sidransky, with Drs. E. Farber (Tulane University), M. Rechcigl and T. Baba, is investigating the effects of nutritional deficiencies on the pathogenesis of hepatomas in rats in an effort to learn if other nutritional deficiencies besides chronic choline deficiency can lead to liver cancer. In short-term experiments, young rats were found to develop signs of acute choline deficiency only when the diet contained no choline and 0.12 percent

or less of methionine. In long-term experiments, no signs of cirrhosis or hepatomas have yet appeared in rats on an adequate choline but methionine deficient diet for 22 months. Species differences have been observed in determinations of liver choline oxidase activity; among nine species, the rat has the highest activity and human the lowest. Pathologic changes in the liver of the rat similar to those seen in kwashiorkor were observed within a few days after tube-feeding of young rats on certain essential amino acid deficient diets.

Dr. H. P. Morris, with Mrs. B. P. Wagner, continues to add to the knowledge of the carcinogenic activity of certain chemical compounds, structural and metabolic relationships, species differences involved, and dietary effects. Last year, Dr. Morris reported that hydroxylation of N-2-fluorenylacetamide (2-FAA) in positions 1, 3, 5, or 7 reduces its carcinogenic potency, and the in vivo hydroxylation that occurs is interpreted by Dr. Morris as a detoxification mechanism or a means of solubilization to facilitate excretion (these compounds occur in the urine in the largest amounts as conjugates of glucuronic and sulfuric acids after 2-FAA feeding). Last year also, it was reported that replacement of the hydrogen atoms on the omega-carbon of the acetyl group in 2-FAA by fluorine led to increased carcinogenic activity. Fluorination of all six omega-carbon hydrogens in 2,7-fluorenylene-bis-acetamide (2,7- FAA) has now been accomplished, and this new compound shows increased carcinogenicity. Sites in the rat most affected were the mammary and salivary glands. It has also been shown that the weak carcinogen, N-1-fluorenylacetamide (1-FAA), was converted into a moderately active one by substitution of the hydrogens of the omega-carbon by fluorine; activity was again most pronounced in the mammary and salivary glands.

Four papers (with Drs. H. L. Stewart and K. Snell) setting forth a large series of observations on the carcinogenic effects in rats of 2, 7-FAA, including comparisons with 2-FAA, have been accepted for publication. Tumors were produced in the small intestine, glandular portion of the stomach and salivary glands, and neurogenic tumors were noted. 2,7-FAA also produces atrophy of many organs and tissues and raises questions anew regarding the role of atrophy in tumorigenesis. Of special interest is the gastric adenocarcinoma produced in the rat, for this is the first instance that this important type of cancer has been produced by an orally administered carcinogen.

Animal experiments concerned with the effects on carcinogenicity brought about by blocking the most active hydroxylation sites in a simple aromatic amine such as aniline or acetanilide have been partially completed. Blocking both the ortho positions and the para position with $-CH_3$, -Cl and -F were studied as well as the para position with -OH.

The 2,4,6-trifluoroacetanilide experiments are still incomplete. Liver tumors were induced in rats fed 2,4,6-trimethylaniline, and a number of animals showed severe cholangiofibrosis. Enlarged pituitary glands were also noted. In the p-hydroxyacetanilide experiment, mammary glands were found to be hypertrophied, and a large number of animals developed enlarged pituitary glands. Enlarged pituitary glands were also seen in rats fed 2,4,6-trichoroacetanilide and in female rats fed acetanilide.

One of the hepatomas (Morris hepatoma 5123) recently developed by Dr. Morris is of considerable significance since its enzymatic characteristics are very similar to those of normal liver and are in fact more similar to normal liver than any other transplantable rat tumor studied to date. Some enzymatic activities are actually greater in the tumor than in normal liver. Dr. Morris is collaborating with investigators in several laboratories in studies on this tumor, particularly in regard to Dr. Van Potter's deletion hypothesis. This hepatoma was produced in the Buffalo strain of rat by a slow acting carcinogen, N-(2-florenyl)phthalamic acid (2-FPA).

Dr. H. M. Dyer, working with Dr. Morris and Miss B. Ensfield, has continued her studies on the interference of 2-FAA and related compounds with tryptophan metabolism. It appears that the excretion of increased amounts of urinary xanthurenic acid, kynurenic acid, and kynurenine derivatives after a load dose of L-tryptophan to rats maintained on carcinogenic concentrations of 2-FAA is due to an inhibition of kynureninase activity. Inhibition may result from combination of the coenzyme pyridoxal phosphate with fluorenamine or some other amino metabolite of 2-FAA in the form of a Schiff base. Therefore, a more than ordinary requirement of vitamin B_6 may be needed by rats ingesting aminocarcinogens. A relatively low concentration of dietary B_6 was found to cause an increase in the induction period and/or a low incidence of tumors of the liver induced by 2-FAA. Low dietary levels of vitamin B_6 inhibited mammary tumor development.

Drs. J. H. and E. K. Weisburger, with Drs. Morris and K. Suzuki and Mr. P. H. Grantham, continue to make many contributions to our knowledge of the mechanisms of carcinogenesis and the chemistry and metabolism of N-2-fluorenylacetamide and related compounds. Previous work had indicated that monohydroxylation is an important biochemical reaction undergone in the body by compounds such as N-2- and N-3-fluorenylacetamide. The suspicion that the more polar metabolites excreted were dihydroxy derivatives has been confirmed through studies performed with the synthesized 2,7-dihydroxyderivatives of N-3-fluorenylacetamide. In addition, N-(7-hydroxy-3-fluorenyl)acetamide formed a sizeable portion of the urinary metabolites and was the only product excreted as a sulfuric acid conjugate.

The 2,7-dihydroxyderivative was excreted mainly as a glucuronide, as was also the previously known 2-hydroxy derivative. The polyhydroxylated compounds possess a number of charged reactive groups which make them especially interesting from the biochemical and biological standpoint.

Studies were continued on the protein binding of 2-FAA and its metabolites. It is obvious that this kind of binding represents a complex series of reactions involving a number of tissue constituents and several metabolites of the carcinogen. Last year, reactions were described that involved a few selected metabolites of 2-FAA, namely, the aminofluorenols, which gave rise to rat liver protein-bound radioactivity as an artifact. Through use of in vitro and in vivo experiments involving proteolytic enzymes, it has been shown that the aminofluorenols form only a small part of the metabolites of 2-FAA and that the artifact type of binding plays only a minor role in the binding noted in in vivo experiments. Studies are continuing on this complex problem.

Protein binding of 2-FAA was studied in rats bearing the Morris hepatoma 5123 with radioactively labeled carcinogen. Since this tumor resembles normal liver in many respects, it was of interest to find that proteins derived from this tumor showed only about 10 percent of the radioactivity of proteins derived from normal livers.

Drs. E. K. and J. H. Weisburger have performed considerable synthetic organic chemical work to prepare compounds suspected of being intermediates in the metabolic sequence, labeled carcinogens and their metabolites for distribution studies, and carcinogens and derivatives for feeding experiments. Not only is knowledge increased in a segment of organic chemistry through such studies, but several new reactions have been developed during the course of these syntheses and many valuable compounds prepared have been made available to several investigators, both within NIH and elsewhere. In addition, physical-chemical data have been acquired by means of ultraviolet and infrared spectra and by measuring ionization constants of aromatic amines. Polynuclear aromatic amines have been synthesized and some show metal chelation.

Last year Dr. H. F. Blum reported on a model that is consistent in a quantitative sense with the existing data on cancer induction by ultraviolet light (a book by Dr. Blum on this subject has been well received by cancer investigators and other scientists). Dr. Blum has now formulated a quantitative model for carcinogenesis by chemical agents. This model, although containing the same fundamental assumptions as the former one, entails equations of a different form, explainable on the basis of temporal relationships of the carcinogenic agent. Preliminary examination of the goodness of fit of the model for data in the literature indicates good approximation. The model also points up difficulties in testing the "specific" effectiveness of a given carcinogen, since multiple factors, which need

evaluation in testing, are involved, some of which are difficult or impossible to measure by the usual testing methods. In particular, the setting of tolerance levels based on the usual type of data obtained in toxicological testing would likely lead to a false sense of security. The new model, like the older one, describes a cumulative, essentially irreversible and essentially nonthreshold process.

Viruses:

The Virus Oncology Section, Laboratory of Biology, has an integrated program of research on viruses and cancer which in toto has a wide variety of experimental approaches aimed at elucidating the role of viruses in human cancer and which, at the same time, permits the individual senior investigators to work with model animal systems of particular interest to each of them.

Virological Aspects of Human Tumors:

A major obstacle to a full-scale attack on the viral aspects of the human tumor problem is the impropriety, at least at the present state of knowledge, of testing the biological behavior of potential human tumor virus preparations in man. Yet, from knowledge of the tumor viruses of laboratory animals, it appears that human tumor viruses, if they exist, are likely to be species specific under most circumstances. Six major approaches, designed to take into account the above difficulties, are under way in the attempt to obtain data relevant to the question of the viral etiology of human cancer: (1) Attempts to detect cytopathogenic or other changes in tissue culture following inoculation of cell-containing and cell-free preparations of human cancer tissues into cultures of cells from human beings and animals; (2) Attempts to detect changes in the chorio-allantoic membranes and allantoic cavities of eggs inoculated with cell-containing and cell-free preparations of human cancer tissues, and study of the phenomenon of viral interference in embryonated eggs utilizing known chicken or human viruses that grow readily in such eggs; (3) Primary inoculation into newborn mice of microsome fractions of human cancer tissues prepared by the methods found most suitable for the Rous sarcoma virus; (4) Tests in newborn mice on nucleic acid fractions derived from human cancer tissues; (5) Procedures for producing immunological tolerance in tests for human viruses involving the inoculation of other animal species; and (6) Electron microscope studies (in collaboration with Dr. A. J. Dalton).

The tissue culture studies, which have been in progress for about two years, have yielded no evidence thus far of a virus of any type being associated with human cancer tissue. A total of 75 separate cancers have been

investigated in tissue culture. With the other approaches, insufficient time has elapsed to expect definitive information. It is of interest to point out that 10 cancer specimens have been employed in coordinated experiments involving all or most of the various approaches listed above.

Because it appears to be of great importance in studies on the possible viral etiology of human tumors to be able to manipulate artificially a virus or host to permit the growth of a foreign virus, Dr. M. A. Fink has been attempting to induce in mice a recognizable, reproducible change by the injection of a known, alien tumor virus (the avian Rous sarcoma virus) in the hope that a useful model system might be developed. Although hundreds of mice have received injections by a variety of routes, no effects have been noted. When attempts to prevent an antibody response in later life were made by injecting the neonatal mouse with a chicken tissue component, with cortisone, or with both, it was found that cortisone, but not the neonatal injection of chicken protein, could completely suppress the formation of antibody to chicken protein. Preliminary results with administration of Rous sarcoma virus to cortisone-treated mice suggest that the virus is affecting the animals adversely, but more careful control of cortisone dosage is needed in further work.

Extracts of 45 human tumors have been inoculated into cultures of human cells and into newborn and feal hamsters by Dr. A. Rabson, and, thus far, no tumors attributable to the inocula have been observed. In one case a cytopathic effect was seen in culture, and the agent producing the effect was adapted to grow in HeLa cell cultures where cytopathic effects were also noted. Attempts at identification are under way.

Dr. S. Stewart is also studying cell-free preparations from human neoplasms. Hamsters were injected when newborn either with fluids from embryonic tissue cultures which had received neoplastic material or with fluids from noninoculated control cultures. A wide variety of tumors resulted in a high percentage of animals (many animals had more than one tumor) not only in the experimental group, but surprisingly also in the control group (essentially the same tumor types with the same incidence). Liver cysts, spleen atrophy, enteritis and polyps of the bowel were also seen in many of the animals. Seven percent of the females developed a trophoblast-like lesion of the uterus. Results obtained in mice which were inoculated for another purpose were similar. Of 276 Swiss mice over 12 months of age that had been inoculated when newborn with culture fluids from control mouse embryo cells, 56 percent developed several types of tumors. In 103 litter mates that received culture fluids plus rabbit antisera (containing antibodies to mouse embryo cells), however, essentially no unusual tumors were seen. Dr. Stewart postulates that the high incidence of neoplasms resulted from an induced tolerance to embryonic cells brought about by the tissue culture inoculum.

Moloney Virus:

Dr. J. B. Moloney continues to add to the knowledge of the murine lymphoid leukemia virus which he isolated from a transplantable Sarcoma 37 that had been transferred to BALB/c mice after 195 tumor generations in strain A/Ln mice. He has now isolated the virus from two other transplant lines of Sarcoma 37, the original tumor line in A/Ln mice and the sarcoma line from which this arose, now being carried in ZBC mice. Further improvements in concentration and purification of the virus have been made by means of protamine precipitation, followed by trypsin digestion of the protamine-virus complex. Regardless of the route of inoculation, the neoplasm produced is always of the generalized type with no tumors at the site of inoculation. The intravenous route produces leukemia deaths in weanling and adult mice about one month earlier than those produced by other routes. The virus is capable of eliciting a 100 percent leukemia response in BALB/c mice inoculated as late as 8 months of age. The potency of the virus has increased with successive passages through newborn mice; after 12 passages the average latent period for 100 percent induction of lymphocytic leukemia has fallen from 6.4 months to 2.5 months period. A high percentage of animals develop the disease as early as 5.5 weeks after inoculation.

Recent studies by Dr. Moloney indicate the following important results: the viral induction of lymphoid leukemia in the rat; a lymphoid leukemia virus that can cross the species barrier; a virus that is not rat strain specific. The agent has not acquired host specificity after repeated virus passage in the rat or mouse, since virus from either rat or mouse passages will induce the disease in both species of animals.

Either thymectomy or splenectomy delays the onset of leukemia in the inoculated hosts although all eventually die with the disease. Thymectomy of the host followed by inoculation results in the induction, in a high percentage of cases, of a Hodgkin's disease-like lesion which is a reticulum cell neoplasm of Type B as described by Dr. T. Dunn. Splenectomy of the host followed by inoculation results, in a few cases, in the induction of myeloid leukemia which can be further differentiated as chloro-leukemia, an extremely rare tumor for this strain of mouse. Cell-free material was prepared from the induced myeloid leukemia and inoculated into intact newborn BALB/c mice; all the mice died with lymphoid leukemia.

Preliminary results give good evidence that the virus preparation tested was free of nuclear material and that the agent is composed of RNA-containing particles. Dr. Moloney has initiated experiments to learn if the nucleic acid isolated by the method of Gierer and Schramm from a leukemic tissue microsome fraction containing active virus can induce tumors.

Preliminary studies by Dr. Moloney indicate that the tumor-producing activity of the leukemic ascites cells, which contain active virus, was completely inhibited by oxidation products of linolenic acid.

Dr. T. Dunn, working with Dr. Moloney, has found that leukemia first appears in the thymus gland in mice inoculated with the Moloney virus. Prior to the development of this lesion there was noted regularly a marked hyperplasia of the spleen and occasionally a localized reticulum cell hyperplasia of the lymph nodes. Transplants of the hyperplastic spleens from mice with no evidence of a true leukemia reproduced the same sequence of lesions observed after inoculation of the virus material, indicating the presence and multiplication of the leukemia virus in a host which showed no overt signs of lymphoid leukemia.

It has been observed earlier that the young mice born to leukemic parents developed "spontaneous" lymphoid leukemias at a high incidence level after a rather extended period of time. Drs. Moloney and N. Ida (M. D. Anderson Hospital, Houston) have undertaken studies to determine the mode of transmission of the Moloney virus to the offspring. Pregnant mice were inoculated intravenously at various stages of gestation. When the virus was given early in gestation (1 to 4 days), a high rate of abortions or resorption of fetuses occurred. A high incidence of leukemia was noted among the young born of the group of mice that had received the virus after the placenta was formed, with average latent periods of 31 to 52 weeks. Thus, at least one mode of vertical transmission of this leukemia agent is through the placental barrier.

Dr. A. J. Dalton, with Drs. Moloney and W. W. Oppelt, has found by means of electron microscopy that in an analysis of Moloney leukemia the primary site of formation and apparently the major source of virus are the megakaryocytes of the bone marrow and spleen. The majority of particles in any one megakaryocyte were in the same stage of development, the stages varying from one cell to the next. Platelets from rats rendered leukemic with the Moloney agent were examined. Many platelets were found which contained particles, occasionally centrally located. Megakaryocytes and platelets of control rats were found to be negative for the particles. The platelets in one case of human lymphocytic leukemia previously treated with an antileukemic agent have been examined and were found to be negative.

Dr. H. Kahler and Mr. B. J. Lloyd, in collaboration with Dr. Moloney, utilizing density gradient centrifugation and electron microscopy techniques, observed that the highest concentration of Moloney virus particles of the 100 millimicron diameter size was found at the level in the centrifuge tube where the density was calculated to be 1.24.

Gain in knowledge of the murine tumor causing viruses would be greatly facilitated if more rapid methods of assay not requiring long latent periods of direct observation in animals were available. Dr. F.J. Rauscher is seeking to improve assay methods by attempts to increase the potency of the viruses under study, reduce or remove inhibitors present in some assay systems, and develop simplified assay, systems, e. g., in embryonated chicken and Japanese quail eggs. One approach showing success is an assay of the Schoolman-Schwartz mouse leukemia virus involving the virus interference phenomenon. The leukemia virus has been serially passaged on the chorio-allantoic membrane (CAM) of embryonated hens eggs. No discrete lesions were observed grossly or histologically; however, when eggs were inoculated with CAM extracts of the first and sixth serial passages and then challenged with influenza virus, a 90 to 99 percent reduction in the titer of the challenge virus was noted. The same CAM extracts when heated to 60°C. for 30 minutes, or extracts of CAM's from eggs inoculated with normal mouse tissue failed to depress the titer of influenza virus. Extracts of CAM's showing this interfering activity have not, however, produced leukemia in mice, as of an approximately 2-months' postinoculation period.

Similar studies are under way with the Moloney virus. It is hoped that these methods can also be adapted to studies with human material as further successes are achieved.

Dr. R. A. Manaker has made additional advancement in the in vitro propagation of the Moloney virus. With improved culture media and primary mouse spleen cultures, he has found that the fluids overlying the cultures are infective for both animals and fresh spleen cultures, provided the cultures are maintained at least two weeks following addition of cell-free extracts of spleen, thymus and lymph node pools obtained from leukemic mice. The virus does not proliferate as rapidly when inoculated into long-term cultures, but will do so sufficiently to produce leukemia in test animals if maintained for as long as three months. Tests for infectivity must be evaluated in terms of the tumor-inducing ability in animals since no cytopathogenic effects are seen in the cultures, even if maintained without transfer for as long as three months. Tissue culture fluids containing the virus may be stored at -70°C. and used to infect fresh cultures at a later date. Leukemia has not been observed in mice inoculated with control spleen culture fluids. On the other hand, leukemia has been observed in uninoculated mothers and uninoculated litter mates of mice infected with active virus material, suggesting that the virus may be contagious. Dr. Manaker's successful propagation of the Moloney virus demonstrates that its in vitro behavior is similar to that of other animal viruses, and the findings add support to the argument that aside from the type of response they elicit in the host, the tumor viruses are not basically different from other viruses.

Drs. Fink and Moloney are investigating the immunological properties of the Moloney virus and have produced the following homologous and heterologous sera: normal mouse and normal rabbit; mouse antinormal spleen; rabbit antinormal spleen; mouse antivirus (formalin-killed); and rabbit antivirus (purified). Only the mouse formalin-killed virus antiserum proved to be effective in neutralizing the leukemogenic activity of the virus. With this antiserum, an indication has been obtained that complement fixation does occur in low titer.

Polyoma Virus:

Dr. L. W. Law, alone and with collaborators, has been particularly active in studying biological parameters of polyoma viruses. With Dr. H. Kahler and Mr. B. Lloyd, Dr. C. Dawe and Drs. W. P. Rowe and J. Hartley (LID, NIAID), Dr. Law has found four strains of virus to be highly variable in a variety of biological properties, including tumor induction, a result not unlike findings obtained with other viruses such as the Rous sarcoma virus and the mouse mammary tumor agent. A highly virulent virus strain required only 20 to 30 particles for tumor formation; on the other hand, another required more than 1 million particles. Different dose levels of the highly virulent strain give different patterns of tumor induction. Dawe's strain adapted for growth on P-388 cells in high-serum medium, undiluted and in 10^2 dilution produces thymic epithelial tumors while in 10^4 and 10^6 dilutions produces unilateral parotid tumors. The nononcogenic strain of Rowe (397), on the other hand, produces parotid tumors only in the undiluted extract.

C57BL/$_{Ka}$ strain mice show a pronounced resistance to tumor induction by a virulent strain of polyoma virus. After an observation period of 6 months, 92 percent of C3Hf/$_{Bi}$ mice have developed thymic epithelial (and other) tumors while none of the C57BL/$_{Ka}$ mice have developed tumors. Only 10 percent of F_1 mice have been tumorous.

Drs. Law, Dawe and Rabson have found differences in infectivity and oncogenic potentialities between polyoma grown on P-388 cells cultured in milk medium and polyoma grown on P-388 cells grown in human serum medium. The former retained high infectivity for mouse and for tissue culture but was nearly nononcogenic, whereas the latter, with a reduced infectivity, remained highly oncogenic. Nevertheless, the virus grown on the milk-adapted cells retained its oncogenicity for hamsters.

Drs. Law and Dawe have observed the appearance of several neoplasms in mice into which polyoma infected fragments of cultured salivary gland tissues (30 - 50 days on a sponge-matrix system) had been placed, both in x-rayed adults or newborn animals. In extensive

studies of three of the tumors, no virus could be detected in two fibrosarcomas, even through eight transfers of tissue in mice, but it was found to be replicating in an epithelial tumor. The results are similar to those reported by Dr. Dulbecco (Cal. Tech.) and by Dr. Sanford (NCI) in their studies of fibrosarcoma development in vitro.

Dr. Law, also with Dr. Dawe, has investigated the effects of x-irradiation, administered prior to introduction of polyoma virus into adult C3Hf/Bi mice. Thirty-eight and 43 percent of the adults presented single or bilateral parotid gland tumors following intravenous introduction of thymotropic polyoma virus, a result strikingly different from that of the same strains infected in the neonatal period. Thymectomy of adults had no influence on the carcinogenic response and virus or x-rays alone were ineffective. X-irradiation plus natural infection (at 3 to 5 months of age) did not induce neoplasms characteristic of this virus. Thymic tumors induced by the virus seldom grew when transplanted into suitable hosts, either in young mice or in x-rayed adults. One of the tumors that did develop was strikingly hormone dependent.

Dr. C. Dawe has obtained additional information as part of the overall study on influences of host factors on the development of cytolytic and proliferative responses to polyoma virus in vitro. With Dr. Law, he has found that cultures of salivary gland tissue from adult mice showed an epithelial proliferative response greater than that seen in cultures of this tissue from newborn mice. At the 30-day interval after infection, cultured salivary gland rudiments from 13- and 14-day old mouse embryos, however showed less epithelial proliferative response than that seen in cultures from either newborns or adults. No proliferative response by mesenchymal cells was seen, and necrosis of these cells was observed in glands of all ages. They have also shown that polyoma virus can induce salivary gland tumors in 50 percent of adult mice receiving total body radiation, even though no such tumors developed in animals treated with x-ray alone nor in adult nonirradiated controls. This information, coupled with early results on the effects of specific antibodies on cultures obtained in collaboration with Dr. W. Rowe (NIAID), plus the knowledge that antibody response is delayed in newborn mice, indicates that the high susceptibility of newborn mice to polyoma virus tumor induction depends primarily on the weakness of the immune response at this age and not on the presence of "primitive" cells or on a higher susceptibility of rapidly growing cells.

Dr. Dawe has shown that response of cultures to polyoma virus of salivary glands from rats, hamsters, mastomys and human fetuses was negative and failed to show any of the changes previously reported in similar mouse salivary gland cultures.

Dr. S. E. Stewart, in collaborative studies with Drs. C. and R. Leuchtenberger (Children's Hospital, Boston), has confirmed and extended previous observations with polyoma virus indicating two main processes in tissue cells of the kidney: a) a degenerative process in the epithelial cells associated with the viral activity leading to destruction of cells, and b) a proliferative process in the stroma without evidence of viral activity, but associated with cellular activity leading to further propagation of abnormal cells resulting in tumors. The epithelial cell changes preceded the proliferation of the stromal cells. It was concluded that only the stromal cells become malignant and, on the basis of histochemical determinations of DNA, that viral proliferation occurred only in the epithelial cells. This result appears to be in accord with Dr. Stewart's previous observations since no renal carcinomas have been found in mice injected with polyoma virus, but renal sarcomas are common. Dr. Stewart suggests that a cell responds to the virus in one of two ways: a) in one instance the cell is used by the virus for its replication, suppressing cell mitosis and finally killing the cell; and b) in the other instance the virus or its products acts as a stimulating agent provoking reduplication of cells resulting in malignancy.

Drs. R. Love and A. Rabson and Miss F. Legallais have determined a sequence of changes in polyoma-infected P388D1 cells grown in culture by means of cytochemical techniques. The earliest abnormalities seen consist of enlargement of the nucleus, nucleolus and nucleolinus and the appearance of intranuclear vacuoles in which normal chromatin and ribonucleoprotein are replaced by abnormal histone-free deoxyribonucleoprotein. Other changes in nucleoprotein become evident in the nucleus, and terminally, alteration in the cytoplasm is observed.

Drs. W. Banfield and C. Dawe have found that virus-like particles can be seen easily in polyoma-induced mouse tumors kept in a maintenance media in tissue culture for as little as 24 hours, but cannot be found after a reasonable amount of searching in tumors examined directly. Several human tumors held in tissue culture from 24 hours to 2 weeks produced negative results. With Dr. W. Rowe (NIAID), initial electron micrographs were made of mouse adenovirus (morphologically in the adenovirus group) and mouse thymic agent (features in common with the Lucké salivary gland, herpes simplex and herpes B viruses).

Drs. M. Stanton has studied the histopathologic characterics of the polyoma-induced osteogenic tumors in Strain A mice. These tumors, which are especially amenable to progression studies because of slow, step-wise changes, are of interest since Strain A mice receiving polyoma virus develop them without developing the variety of tumors observed in other strains and without affecting the incidence of spontaneous pulmonary alveologenic tumors.

Dr. A. Rabson has succeeded in producing tumors of the lung in hamsters by direct instillation of polyoma virus in the trachea. In 6 of the 16 animals autopsied lung tumors with histologic features of bronchiolar carcinoma, alveolar cell carcinoma and squamous cell carcinoma were found. The squamous cell carcinoma, which appeared to arise in bronchioles and intra-pulmonary bronchi, has been established as a transplantable tumor and is now in the 16th transplant generation. Attempts to isolate polyoma virus from this transplantable tumor and from one of the primary tumors have been unsuccessful, an experience similar to that of other investigators who have encountered difficulties in isolating this virus from the sarcomatous lesions of hamsters.

Newborn Mastomys (Rattus natalensis) inoculated with polyoma virus by Dr. Rabson developed tumors in 14 of 17 animals autopsied. Thus far, no parotid gland or thymic tumors similar to those produced by polyoma virus in mice have been observed, however, Polyoma virus was isolated in tissue culture from suspension of one of the renal sarcomas that developed.

Mammary Tumor Agent:

In a study designed to elucidate the factors responsible for the sudden disappearance of the mammary tumor agent from a few strain RIII mice, Dr. H. B. Andervont has obtained further evidence that the virus carried by RIII mice is much weaker in tumor-inducing capability than that carried by C3H mice, thus being one factor of probable significance in the disappearance of the agent. The observation that an appreciable incidence of tumors occurred in F_1 hybrids derived from agent-free (RIII-) females and agent-carrying (C3H+) males was of interest.

RIII virus has been introduced into C3H mice and C3H virus into RIII mice by foster nursing, and the animals have been followed now for several generations of inbreeding. The C3H mice had a low incidence of tumors, and no definite signs of increased potency of the weak RIII virus were seen. With the RIII mice carrying the C3H virus, observations indicate that a single strain of virus in hosts of the same genetic constitution shows a pronounced variation in its ability to induce tumors. Even within a highly susceptible family, an occasional RIII mouse is very resistant even to the potent C3H agent.

In some cases, tumor-causing viral agents can escape through certain sized pores in the membranes of diffusion chambers placed in the peritoneal cavity of mice. For examples, Dr. R. Merwin has shown that the mammary tumor agent passes through pores 0.45 micra but not 0.10 micra in diameter, and that the Moloney virus passes through 0.45 micra pores, but no smaller

ones. Even with 0.45 micra pores, no tumors appeared in mammary tumor agent-free BALB/c hosts when mammary tissue from agent-free C3H donor mice was placed in the chambers, a finding providing additional evidence that the virus is indeed absent from so-called virus-free mice.

Rous Sarcoma Virus:

In attempting to determine the ultimate limit to which the concentration of Rous sarcoma virus in sarcoma tissue can be increased through continued selective serial passage in chickens, Dr. W. R. Bryan has made such passages, selecting the tumor first to appear in each batch of 40 inoculated chickens, for the past 5 years at biweekly intervals (except during the past year the interval was changed to monthly). By this procedure the virus yield of tumors has been increased about 100-fold, but during the past year little increase was observed. It appears unlikely that the yield of virus will be increased further by this technique, but the virus concentration already achieved justifies resumption of studies on purification of the virus.

Drs. Bryan, H. A. Sober, and F. J. Rauscher and Mr. J. P. Kvedar are attempting to purify further the Rous sarcoma virus obtained from "high potency" tumors by means of differential ultracentrifugation and chromatography on cellulose ion-exchange columns. An increase in virus activity, similar to the 100-fold increase obtained by the selective passage procedure, is present in partially purified fractions separated by differential centrifugation or cellulose chromatography. By both procedures there is a 2- to 3-fold increase in viral activity per gram equivalent of tumor tissue, indicating that an "inhibiting" factor is removed.

Drs. Rauscher and M. A. Fink have studied the effects on the production of infective Rous sarcoma virus (RSV) by conditioning the host with Freund's adjuvant. When the adjuvant was given to young chicks before virus inoculation, extensive granulomatous lesions developed at the site of injection (subcutaneous); when a small dose of RSV was injected into these lesions, the granulomas grew at an accelerated rate and appeared to invade adjacent muscle. Extracts of these growths yielded 2 to 4 logs of virus. The same small dose of virus failed to produce tumors in control birds pretreated with saline, and RSV could not be demonstrated in tissue extracts from these control animals. The incidence and severity of metastases were also increased in birds pretreated with adjuvant. When chicks were inoculated with a dilution of RSV that failed to produce tumors and were subsequently inoculated with adjuvant, 85 percent developed relatively large amounts of anti-RSV antibody in their sera. The sera of control negative birds challenged with saline remained free of significant levels of antibodies.

Although Dr. Rauscher has failed to effect a recovery of infective RSV from RSV-specific antibody complexes by treatment with fluorocarbon (Genetron 113), he has done so by techniques of ultracentrifugation and under certain conditions with simple dilution. Through special techniques involving trypsin he has also made noninfective lesions produced by low doses of RSV or noninfective lesions in Japanese quail eggs yield infective RSV.

These techniques that are opening leads for enhancing host response to a virus, for preparing more potent virus, for detecting and eliminating inhibitors, and for measuring responses produced by minimal amounts of virus are particularly important for the study of the initiation and course of viral diseases as they exist in nature and are especially germane in laying the groundwork for investigation of the viral etiology of human tumors.

Other Virus Studies:

In collaboration with Dr. J. A. Reyniers (Germ-Free Life Research Center, Tampa, Florida) and Mr. J. P. Kvedar, Dr. Rauscher is evaluating the value of Japanese quail (Coturnix coturnix japonica) embryonated eggs and posthatched chicks in virus and cancer research. Their small size before and after hatching, their ease of maintenance, and their rapid generation time makes them especially suitable as an avian host in studies requiring long periods of observation, particularly since Dr. Rauscher has shown that they may be manipulated in a manner similar to chickens and that they are susceptible to at least 6 different viruses. In addition, certain interesting differences have been noted with the Rous sarcoma virus and with the visceral lymphomatosis virus. Of special interest is the finding of a substance inhibitory to the Rous sarcoma virus growth in eggs that failed to develop gross lesions. The possible relationship of this phenomenon to "interferon," to the resistance-inducing-factor of Rubin, and to lymphomatosis virus is under study.

More knowledge is needed on the indigenous mouse viruses because, among other reasons, they may interfere with the expression of tumor-causing viruses under study, they may produce disease in colonies of study animals thus invalidating experiments in progress, and they may, if sufficient information can be gained, serve as models of tumor virus behavior involving latent infections. Dr. R. A. Manaker has discovered three such viruses (two that produce hepatitis and one that produces pneumonia) and has determined several of their characteristics. Such information is useful in the control of the diseases produced in colonies by these viruses and provides basic knowledge needed for study of the tumor viruses in animals that are potential carriers of these agents.

Drs. C. Dawe and L. Kilham (DBS) did not find a proliferation-promoting effect of rat virus in organ cultures of rat salivary glands, a system analogous to the polyoma virus-infected mouse salivary gland culture system.

Drs. W. Banfield and G. Ramirez and Mrs. D. Brindley, in studies with a spontaneous reticulum cell sarcoma in a hamster now carried through 12 passages, have shown that while injections of blood, ascites fluid, and brain from tumor-bearing animals produce tumors in the recipient hamster, cell-free filtrates of brain, ascites fluid, and tumor so far have not. Of 6 animals fed fresh tumor tissue, 1 developed systemic tumor involvement and another laryngeal occlusion from submucosal tumor growth. More remarkably, of 10 untreated animals caged with 10 animals inoculated with tumor, 6 died with laryngeal obstruction caused by submucosal tumor growth and 2 killed animals had systemic tumor involvement. The tumor also arose in 1 of 3 hamsters separated by a screen from tumor-bearing animals. As yet, no virus-like particles have been seen upon examination by electron microscopy.

In electron micrographs, Dr. R. F. Zeigel has observed type A "virus particles" of Bernhard budding from the apical plasma membrane of pancreatic acinar cells in the chick. Although the viral nature of these particles has not been established, they possess morphological characteristics closely similar to those described in association with lymphomatosis, Rous sarcoma, myeloblastosis and other viral infections. When particles were observed to bud from pancreatic acinar cells, all other tissues examined contained the particles in intercellular spaces and in intracytoplasmic vesicles and vacuoles. When absent in the pancreas, they were not seen in other tissues. In a study of the albumin-secreting gland of the hen oviduct, the secretory product appears to be produced in close association with the ergastoplasm, accumulated in large cisternal regions and released into the secretory lumina.

Mr. B. G. Young and Dr. P. Mora are conducting fundamental studies on bacteriophage and are preparing to apply some of the techniques developed to the tumor-causing virus, polyoma. Such studies are part of a program designed to study interactions of biological system at the molecular level and employ characterized, synthetic positive (basic) or negative (acid) derivatives of polysaccharides with different degrees of branching and charge distribution (vide infra). The bacteriophages are being used as model systems to study quantitatively the changes that polyelectrolytes may bring about in the surface proteins of the virus, in order that more can be learned of effects on tumor-causing viruses, particularly in regard to blockage of the infective process.

The extent of inactivation of viability of bacteriophage (by blockage of attachment of the phage to the bacterial cell and/or the injection of DNA into the bacterial cell) was determined by means of incubation of the virus with negatively charged synthetic polysaccharide derivatives. Inactivation of bacteriophage by polyglucose sulfate, which occurs at an acidic pH range in which sulfuric acid does not produce inactivation, was not reversed by further incubation at neutral pH, by increasing the salt concentration, or by adding positively charged macromolecules. Thus, the inhibition is not due to a purely electrostatic interaction with the positive lysozyme-like protein in the tail of the phage. That an ion exchange effect was not producing the inactivation was shown by inactivation even in the presence of high calcium ion concentrations. Furthermore, since polyglucose sulfate, labeled with S^{35}, interacted with the T2 bacteriophage directly and did not penetrate into the E. coli cell, the mechanism of inactivation is most likely due to prevention of normal attachment or DNA injection, rather than blocking processes of viral multiplication in the cell.

When the inactivation was only partial, the residual viable phage had a normal attachment rate to the E. coli. The attachment of the total phage components also was not reduced significantly. These conclusions were based on measurements done with P^{32}-labeled DNA and S^{35}-labeled protein of the phage. Experiments of shearing off the labeled attached phage from the E. coli surface by swirling it in a Waring blendor showed that it was easier to shear off DNA from the inactivated phage than from an untreated virus. No incorporation of labeled P^{32} into the progenies of the bacterial phages was found.

A reasonable interpretation of the above results is that the lysozyme-like protein is blocked by the anionic polysaccharide, but not sufficiently to prevent attachment, only the penetration and injection of the DNA. It is possible that a second inactivation mechanism might also occur, possibly simultaneous with the other mechanism. Negative DNA in the head of the virus is known to be partially neutralized by positive oligopeptides such as putrescine (findings of Dr. B. Ames, NIAMD). The DNA may change its shape if the positive putrescine is removed by the negative polysaccharide, making eventual passage through the narrow tail core impossible.

It is of interest to note that a small percentage of the bacteriophage population which remained viable gave progenies that were more resistant to further inactivation, suggesting that inactivation might be a useful tool for genetic studies. Low concentrations of polyglucose sulfuric acid were also found to reactivate some of the bacteriophage that had been inactivated with antibody, indicating a direct interaction between the antibody and the polyglucose sulfate. Such findings suggest possibilities of antibody fractionation and new ways of studying antigen-antibody interactions.

In the course of preparing for studies on tumor viruses, Mr. Young and Dr. Mora are modifying the method of Dulbecco and Sachs, which produces plaques in a monolayer of embryonic tissue culture, for studies on polyoma.

Host Genetics in Carcinogenesis:

Genetics of the mouse has reached a state of development where analysis of effects of specific genes can now profitably be pursued. There are at least 87 gene loci of the mouse identified in 19 linkage groups, and they may represent all but one of the chromosomes. Nine of these loci have been shown to be linked with neoplasia. Not only has Dr. W. E. Heston continuously over the years made major contributions to the knowledge of strain differences in relation to cancer, but he has been in the forefront in amassing data on the role of specific genes of the mouse associated with cancer. He has been particularly interested in two loci that are linked with cancer: the obese gene ob in linkage group XI and the lethal yellow gene A^y in linkage group V. The ob gene results in excessive obesity, and the A^y gene is lethal when homozygous, but when heterozygous causes the coat to be yellow and causes an increase in body size. From studies on the latter a unique system has been developed which will permit new approaches to understanding of the mechanism of gene action in mammals, at both the molecular and higher organismic levels. Since the A^y gene is lethal when homozygous, forced heterozygosis at this locus during the long period of inbreeding in the YBR strain has led to a situation in which F_1 hybrids resulting from outcrosses between mice of the YBR strain and those of any other inbred strain are isogenic in that they are genetically alike except that half carry the A^y gene and are yellow and half carry the nonagouti allele and are nonyellow. Thus, in these F_1 hybrids, analysis can be made of the effect of this specific gene, for any difference in occurrence of tumors in the two color types is known to be due to this gene. A further advantage in these F_1 hybrids is that tissues can be transplanted freely between the two color groups since in Dr. Heston's Laboratory it has been shown with skin transplants that the A^y gene has no histocompatibility effect. This enables investigators, after identifying an effect of the gene on any type of tumor, to try further to identify its path of action through transplantation of tissues or organs.

Dr. Heston is employing this system with transplantation of ovaries to learn if these organs are involved in the mechanism of action of the A^y gene since the gene reduces the age at which mammary tumors appear in virgin females from 15 to 8 months and since breeding eliminates the difference between groups of female mice with and without the gene. Transplantation studies in young adult animals so far have failed to implicate the ovaries; transplantations in day old F_1 hybrids are being done. If these investigations give negative results Dr. Heston will seek other pathways of gene action through other transplantations.

In addition to the increased susceptibility to mammary tumors and pulmonary tumors previously reported, last year Dr. Heston found that males with the A^y gene had a significantly higher incidence and average number of spontaneous hepatomas than those without the gene. The feeding of carbon tetrachloride had no detectable carcinogenic effect on inducing hepatomas in these highly susceptible male mice. In these F_1 hybrids the A^y gene increased body weight by about 10 grams in the males and 15 grams in the females. Positive correlations were found in the males between the number of hepatomas and body weight, body length and length of femur.

In similar studies on the ob gene, Dr. Heston has found that this gene decreases the occurrence of spontaneous pulmonary tumors. He previously reported that this gene reduces the incidence of induced pulmonary tumors. Male mice with the ob gene also show a higher incidence of hepatomas, and administration of carbon tetrachloride gave no differences in occurrence of hepatomas between the obese ob mice and the normal sibs.

Dr. M. K. Deringer previously reported a reduction in the incidence of mammary tumors in mice of strain C3He which she produced by transplanting fertilized C3H ova to pregnant uteri of C57 black mice without the mammary tumor agent and an even lower incidence in DBA/2e mice (transfer of fertilized ova from strain DBA/2 to C57 BL uteri). Although the data are not complete, it appears that forced-breeding raises the incidence of mammary tumors in strain DBA/2e females, but the incidence does not begin to approach the nearly 80 percent figure seen in the DBA/2 strain of breeding females.

In studies of F_1 hybrids resulting from outcrosses of strain YBR animals (carrying the lethal-yellow gene) and strain DBA/2 animals (susceptible to the development of leukemia) in which the skins were repeatedly painted with 3-methylcholanthrene solution, Dr. Deringer has, as yet, found no effect of the lethal-yellow gene on the development of leukemia, but only a small number of leukemias have thus far been observed. To date, tumors of the skin occurred more frequently in the yellow than in the brown females and about equally in the yellow and brown males of the treated groups.

Radiation Studies:

Dr. L. Law is continuing his investigation of the role of variables in x-ray induced and spontaneous leukoses. Chimeras developed in C3H mice (both the Law and Bittner sublines) through introduction in the neonatal period of AKR bone marrow showed the same incidence of x-ray induced lymphocytic and other neoplasms of reticular tissue as the nonchimeric controls, but introduction of AKR thymic tissue into the chimeras increased the frequency of tumors in the Law subline, but not in the Bittner subline. Few thymic grafts were involved in the neoplastic response, and the tumors grew in the host strain ($C3Hf/L_W$) and not in AKR mice. The presence of C3H thymus may therefore inhibit the process of leukemogenesis.

Fetal C57 BL mice were resistant to doses of x-ray that induced tumors in 24 hour- and 1 month-old mice. This finding is in agreement with that of Upton, who also observed an insensitivity of fetal tissues in another strain, and indicates the desirability of further investigation, particularly during those early periods when other tissues function as hematopoietic tissues.

Total body irradiation increases the incidence of reticulum cell sarcomas and granulocytic neoplasms in thymectomized C57 BL mice. Shielding of a thigh, in contrast to this influence on thymic lymphoma induction, has been observed to be without effect on the induction of these later appearing forms of leukoses. Differences in response to fractionated, whole-body x-irradiation have been observed among three C3H sublines.

Miss D. Uphoff has extended her work on the genetic factors influencing protection against lethal total-body irradiation by a postirradiation inoculation of bone marrow to include the mid-lethal dosage level. Sufficient data are now available so that generalizations and comparisons can be made as to the effects of the dose of irradiation on the successful transplantation of bone marrow at the mid-lethal and lethal dosage levels. The postirradiation inoculation of genetically compatible marrow (marrow donor and host share the same histocompatibility -2(H-2) phenotype) will result in protection of the irradiated mice with no apparent immunological response at either dosage level. The available information indicates that these protected animals are chimeras consisting of an irradiated host of one genotype and a hematopoietic system of the donor genotype. The inoculation of genetically incompatible marrow (marrow donor and host of different H-2 phenotypes or of the H-2^k phenotype), gives somewhat different results. Following lethal irradiation incompatible marrow may give rise to a graft versus host reaction ("secondary disease") which may result in death of the irradiated marrow inoculated mice after apparent recovery from the acute irradiation damage. The severity of the reaction and degree of mortality varies with the strain combinations and is probably a function of the antigenic incompatibility of the strains employed. The inoculation of genetically incompatible marrow following exposure to a mid-lethal dose of irradiation may result in what appears to be a host versus graft reaction which may result in an early death of the treated mice. Although the exact cause of death has not been determined, it is clearly a function of the phenotype of the irradiated host. It occurs following the irradiation and marrow inoculation of two different strains of the H-2^k phenotype (other strains of the H-2^k group have not been tested). This lethality of the treatment has not been observed in irradiated hosts of the H-2^a, H-2^b or H-2^d phenotypes.

Miss Uphoff has obtained chimeras by exposing mice to lethal doses of x-ray and protecting them with suitable bone marrow. Under these conditions the lymphomas arising in the irradiated mice were mostly of the donor

tissue, and those of the host genotype were cases where initial protection by donor marrow permitted regeneration of the host's marrow. When mice received a mid-lethal dose of irradiation and homologous bone marrow, most tumors were of host origin. At this dose level lymphomas arise primarily in genetic combinations in which the host and donor are of different histocompatibility-2 (H-2) phenotypes. In H-2^b or H-2^d combinations in which host and donor are of the same H-2 phenotype, lymphomas have not been observed. It would appear that although the marrow graft is capable of protecting the host against the acute irradiation damage, it does not appear to protect the irradiated mouse against this late radiation effect, possibly because of the regression of the graft as indicated by the lymphomas of host tissue origin. The demonstration of a "tiding over" effect of genetically incompatible marrow at this dosage level may be an experimental counterpart to the clinical observations on the marrow inoculation in the Yugoslavian radiation accident victims in which there appeared to be clinical improvement, but subsequent disappearance of donor-type cells from the circulating blood, indicating a regression of the "graft."

Miss Uphoff had found earlier that amethopterin may protect mice that would normally succumb to a homograft reaction following lethal, total-body irradiation and homologous marrow inoculation. Such mice have a permanent state of immunologic nonreactivity since it was shown that the mice are chimeras. On the basis that treatment with amethopterin in the presence of a specific antigen might lead to a "tolerant" state in the adult mouse, mice were treated with the drug and a strain specific antigen in the form of a crude tissue brei of spleen and thymus. Effects were determined by transplantation of tumors of the same strain as the antigen. Preliminary results are suggestive of success in inducing a "tolerant" state since 5 of 15 mice receiving both drug and antigen died of progressive tumor growth; mice treated with antigen alone were resistant to tumor growth, while with drug treatment alone only 1 of 14 had a positive test. Because of the urgent clinical need for a method other than irradiation for the preparation of the host for a homotransplant, studies on dogs are under way (with Drs. Ferrebee and Thomas, Mary Imogene Bassett Hospital).

Radiation of Cells in Tissue Culture:

Dr. M. Elkind, in an extension of his previous studies dealing with the repair of sublethal x-ray damage, has found certain experimental conditions under which it appears that survival of mammalian tissue culture cells following irradiation may be influenced by the proximity of neighboring cells. Such a result has important implications since one of the basic tenents of radiobiology, the assumption that the site structure in cells lethally affected by ionizing radiation is discrete, requires that a cell in a group of cells should survive as an individual and uninfluenced by the proximity of its neighbors.

In an investigation of the influence of drugs on lethality and recovery in x-irradiated mammalian cells grown in culture, Drs. Elkind and W. Mohler have obtained results with 5-bromodeoxyuridine (BUDR) which indicate: (1) BUDR can increase the x-ray sensitivity of a Chinese hamster culture cell line; (2) the increase is accompanied by alterations in both survival parameters (mean lethal doses (MLD) and extrapolation number); (3) the effects on MLD and extrapolation number may be separable under some circumstances; and (4) the alteration of sensitivity is probably not merely the result of nonspecific cell injury since other drugs alter one x-ray survival parameter (MLD) without affecting the other.

Immunological Phenomena and Polysaccharide Studies:

Improvements in techniques and increase in knowledge of immunological approaches are opening new avenues of research on host humoral and cellular factors involved in natural immunity to infection and neoplastic disease. The polysaccharide nature of bacterial endotoxins, their importance in study of immunologic phenomena, and their ability to damage neoplasms (a matter of long-standing interest in the Institute stemming from the early work of Dr. M. J. Shear), plus the increasing knowledge of the production by the body of a number of substances, including antibodies and special enzymes, in response to infection and tumors have led to expansion of programs in the Laboratory of Chemical Pharmacology in the Polysaccharide Section.

Endotoxin Studies:

Bacterial endotoxins, polysaccharide materials now prepared by Drs. M. Landy and E. Ribi (NIAID) in highly potent form with no more than 0.5 percent nitrogen and 1.7 percent fatty acid ester, are highly useful materials in the study of immunity, and with these and the highly sensitive immunological techniques being developed by Dr. Landy and his associates, certain phenomena exhibited by the whole organism and cells that were puzzling in the past are now becoming clear. The nature of the effects of endotoxin on mammals is under study in several laboratories, including the various means by which host susceptibility can be modified. Although intensive investigation is under way on the state of refractoriness or tolerance produced by prior treatment of an organism with endotoxin, kinds of treatment that greatly increase susceptibility are less well defined. These latter are: reticuloendothelial system-blockade, adrenalectomy, administration of BCG vaccine, and tumor implantation.

In preparation for more extensive work designed to learn whether these diverse treatments produce in the host any change in common, Drs. Landy and R. J. Trapani, in collaboration with Dr. E. Suter (University of Florida), have investigated the possibility of additive or synergistic effects of a

combination treatment of CAF_1 mice with BCG vaccine and implantation with sarcoma 37. Results were determined following subsequent challenge with Sal. enteritidis endotoxin. No synergistic effect was found; the effects were not additive, and the results indicated interference of the action of BCG by the tumor.

In the course of studies on alterations in the somatic endotoxin complex of Gram-negative bacteria that occur during the serum bactericidal process (see last year's report,) it was observed that very small amounts of antibody sufficed for bactericidal activity. Dr. Landy, with Drs. J. Whitby (guest scientist) and J. Michael (Visiting Scientist), has developed a simple, specific and objective assay for bacterial antibodies that is much more sensitive than previously developed procedures. Tests with immunologically calibrated antiserum against Sal. typhosa showed that the smallest amount of antibody measurable by the method was 0.0002 microgram antibody and that 0.001 microgram provided marked bactericidal effect. On this basis normal mouse serum was estimated to contain approximately 0.01 to 0.02 microgram antibody nitrogen (against Sal. typhosa) per milliliter.

In a careful study of the pyrogenic response of the rabbit to endotoxin, Drs. Landy and W. Keene (PHS Hospital, Boston) have also developed a more precise bioassay for endotoxin by means of a procedure requiring administration of amounts of endotoxin that will produce febrile responses that fall on the linear portion of the dose response curve.

The inactivation of endotoxin by blood plasma described in previous annual reports was found to have characteristics of an enzyme-catalyzed reaction. The progress of this reaction was followed by measuring residual substrate (endotoxin) concentration in the pyrogen assay, and the assay was based on the assumption that the residual fever-producing substance in the plasma-endotoxin reaction mixture was unaltered endotoxin. In the previous report, it was suggested that the residual pyrogen of incubated plasma-endotoxin reaction mixture had the pyrogenic properties of endogenous serum pyrogen. With Dr. Keene, Dr. Landy has now demonstrated that animals tolerant to endotoxin are also tolerant to the residual pyrogen. In addition, daily injections of the residual pyrogen induced endotoxin tolerance. Since the most distinctive quality of endogeneous pyrogen is that it is equally pyrogenic for normal and endotoxin-tolerant animals, it appears that the residual pyrogen of incubated plasma endotoxin reaction mixtures is not endogenous pyrogen.

Not only is endotoxin inactivated by blood plasma, but tissues are also capable of inactivating endotoxin. As in the case of the inactivating system in blood plasma, the in vitro inactivation of endotoxins by cell-free homogenates of perfused rabbit liver also appears to be an enzyme-catalyzed reaction, but unlike the former is not influenced by calcium ions (studies by Drs. Landy and Trapani with Dr. V. Waravdekar-AFIP). Preparations

derived from rabbit liver display good endotoxin-degrading activity, but several other tissues from rabbit showed extremely low or no activity. Extracted cells also yielded clear preparations low in nitrogen and total solids that were potent in destroying pyrogenicity and tumor-damaging activity of bacterial endotoxin. Rabbit granulocyte homogenates were likewise found to be effective by Drs. Landy and A. L. Notkins in activating endotoxin, although other formed elements of blood, including human erythrocytes, platelets and rabbit monocytes were not found to inactivate endotoxin. Administration of large amounts of endotoxin to mice did not lead to elevation of serum lactic acid dehydrogenase, an intracellular enzyme that might be expected to be elevated in the serum if the endotoxin were producing damage to granulocytes as has been claimed in reports in the literature. Furthermore, no release of this enzyme was detected when mouse peritoneal macrophages were incubated with endotoxin.

Drs. M. Woods and Landy have shown that the capacities of a series of endotoxins (from different Gram-negative genera, isolated by different procedures, and having different potencies in eliciting characteristic effects in the host) correlated well with stimulation of glycolysis in vitro. Moreover, experiments on the degradation of endotoxin by humoral and intracellular enzymes of the host, or by progressive acid hydrolysis, resulted in a loss of capacity to stimulate glycolysis. Both aerobic and anaerobic glycolysis are stimulated; the lowest effective concentration of endotoxin is about 0.0001 microgram per ml., and 0.3 microgram per ml. generally provides maximal effect. Similar effects could be produced in vivo as measured with in vitro determinations of glycolytic activity of peritoneal macrophages obtained from mice given endotoxin a few hours previous to the isolation of the cells.

Tumor Cytotoxic Factors in Serum:

Systematic investigation of the antibody-complement system in normal serum lethal to mouse tumor cells, which was reported on last year, has been largely completed by Drs. Landy, Trapani, Michael and Woods. The general findings are that normal sera from many animal species exhibited in vitro a lethal effect on ascites cells of sarcoma 37 and a considerable number of other tumor cells of mice. This in vitro activity of serum on the tumor cells of a heterologous species was manifested by: failure to proliferate in a susceptible host; altered permeability to eosin; morphologic changes; and cessation of glycolytic activity. The system in serum responsible for these effects consisted of a natural antibody and complement. This antibody had specificity for an antigen present in tumors and lymphoid tissues of mice. In continuation of the study, the failure of fresh human serum to exert a comparable effect on tumor cells in vivo was shown to be due to uptake of the antibody

by normal lymphoid cells of the mouse. In tests for this cytotoxic activity, absence of the cytotoxic effect was found in 7 of 17 species studied. Further evaluation indicated, however, that the class of "inactive" species did in fact possess a functional antibody and complement against a bacterial component. Furthermore, sera from two "inactive" species, such as hamster and guinea pig, when combined would give an active preparation as measured by red cell lysis in an appropriate system. It is therefore evident that serum from various species may differ strikingly in the properties of complement they contain.

In collaboration with Dr. R. Smith, the levels of cytocidal activity (mouse ascites tumor test systems) in the serum of cancer patients was compared with that in normal human serum by Drs. Burk and Woods. The action of such sera (active and 52°C. -inactivated) on the patients' own cancer cells was also investigated. Preliminary results indicate somewhat higher than normal levels (2-to 3-fold) of cytocidal activity in the sera of cancer patients.

Antigens Against Human Carcinomas:

The complexities encountered in the study of host humoral and cellular factors in natural immunity to infection and neoplastic disease and the critically sensitive techniques required for such investigations are well illustrated in the experiments summarized above. Perhaps for these reasons more than any other, progress in immunology and cancer has been slow despite a vast amount of effort expended in this direction over the past fifty years. With such modern procedures, Dr. B. Björklund (State Bacteriological Institute, Stockholm) has conducted experiments which led to a recent report that human carcinomas have antigenic properties different from those of normal human tissues. By prolonged immunization of horses with preparations of pooled human carcinomas, an antiserum cytotoxic for HeLa cultures was obtained. This cytotoxicity, which was not affected by absorption with normal human tissues, was removed by absorption with human carcinoma tissue or by HeLa cells.

Drs. Landy and Trapani, in collaboration with Dr. Björklund have initiated investigations which not only sought to confirm the principal findings of Björklund, but also were designed to explore the possibilities of developing serological procedures for more extensive work with such antigenic preparations. Their serological experiments have indicated that it is possible, under appropriate conditions, to "coat" tannic acid treated erythrocytes with preparations extracted from HeLa cells with buffer at pH 8. The treated erythrocytes were agglutinated to high titer by horse antiserum against pooled human carcinomas. It was suspected that the antiserum also contained antibodies against normal tissue antigens. The presence of such antibodies was demonstrated with the use of tanned erythrocytes coated with pooled

normal human serum. These cells were also agglutinated to high titer by the horse antiserum. Practically all of the antibodies reactive with human serum coated erythrocytes could be removed from the antiserum by absorption with cells suspensions of normal human tissues. However, the reactivity of such absorbed serum with HeLa antigen-coated erythrocytes, and its cytotoxicity for HeLa cultures, were not discernibly affected. On the other hand, analogous absorption of the antiserum, with Björklund's antigen preparations derived from human carcinomas, removed cytotoxicity for HeLa cultures and reactivity for erythrocytes coated with HeLa antigen; it is noteworthy that reaction with erythrocytes coated with normal human tissue antigens remained unchanged. The absorption technique was controlled by parallel treatment of an antiserum prepared in rabbits by immunization with normal human serum. This antihuman serum was cytotoxic for HeLa cells and agglutinated erythrocytes coated with HeLa antigen or with preparations from normal human tissues. However, these properties were no longer evident after the serum had been absorbed with normal human tissues.

Immune Tolerance Induced by Breeding:

Last year, Drs. M. K. Barrett and E. J. Breyere (Research Fellow) reported the interesting finding that a state of immunological tolerance can be induced in female mice by breeding with males of another strain which makes them susceptible to transplants of tumors and skin from the strain of the male with which they were bred. Further details have been developed, and, to date, the effect has been observed in 3 genetic systems with 3 transplanted tumors and with skin homografts in 2 combinations. The H-2 histocompatibility locus does not appear to be involved. This tolerance is highly specific and depends upon a genetic concordance between the transplanted tissue and the sire of previous litters. Tolerance was not found in females parous from intrastrain matings, nor in virgins, nor in those parous by males of still another strain unrelated to the graft. Experiments under way have thus far shown the following: (1) Tolerance increases with multiparity. (2) It is effective both in the case of "natural" and induced resistance. (3) It is long lasting, i.e., at least 150-200 days in the mouse. (4) A previously induced immune state can be partially abrogated by specific parity. (5) This tolerant state is resistant to the usual immunization procedures, i.e., immunity cannot be induced in tolerant females by an inoculation of homologous blood and a degree of tolerance remains even after the rejection of a homologous skin graft. (6) The effect of parity is not due to mere exposure to the male in caging, and (7) the presence or absence of the mammary tumor milk agent in the male had no detectable influence.

Polysaccharide Investigations:

The in vivo production of cytoplasmic vacuoles in ascites tumor cells following the intraperitoneal administration of polysaccharide was

reported previously by Dr. M. Belkin. The phenomenon, which occurs only in vivo, appears to be the result of an antigen-antibody reaction. In collaboration with Dr. Dalton in studies of treated cells of three ascites tumors, the following results have been observed with electron microscopy:

1) In sarcoma 37 cells the vacuoles appear to arise primarily at the site of virus particle formation; but others arise also from preformed small vacuoles and "inclusion bodies" which are occasionally present.

2) Vacuoles develop in Yoshida ascites cells from preformed small vacuoles and membrane-enclosed, electron-dense inclusion bodies.

3) In Ehrlich ascites cells, few preformed vacuoles are present in untreated cells, and the vacuoles develop more slowly from membrane-bound, electron-dense "inclusion bodies"; the cells of this variant of Ehrlich ascites tumor were rarely found to contain virus particles.

The programs of the Macromolecular Chemistry Section, Laboratory of Chemical Pharmacology, have as their over-all aim study of the interaction of biological systems on the macromolecular level, with special emphasis on the forces that initiate the interactions and that orient the interacting biological systems in a manner of high efficiency. Included in these programs are studies on polysaccharides, and important information about these and related compounds complement the results of investigations made by Dr. Landy and his group, often obtained in collaboration.

The extensive collection of polysaccharides synthesized by Dr. P. T. Mora and Mr. J. W. Wood, which includes polymers of various sugars and their substituted derivatives with negative (acid) groups (such as polyglucose sulfate or carboxyl derivatives), has been enlarged now to include derivatives substituted with positive (basic) groups. By synthesizing amino derivatives, they have obtained polymers with graded substitution of positive groups. In the course of the work, new methods were developed which led to the introduction of unprecedentedly high amounts of amino groups into polysaccharides: (1) The reduction of the tri-beta-cyanoethyl derivative of polyglucose with lithium aluminum hydride in tetrahydrofurane, and (2) the reaction of 1-N-diethylamino-2, 3-eposypropane with polyglucose in aqueous sodium carbonate solution. Some of the synthetic polysaccharides (polyrhamnose and polymaltose) possess considerable lipemia-clearing activity without demonstrable toxic or anti-coagulant activity, and some others inhibit mitosis in tissue culture (findings of Professor R. Meir, Ciba, Ltd., Basle and Dr. A. Di Marco, University of Milan, in collaboration with Dr. Mora). These synthetic, charged polysaccharide derivatives

can also be usefully employed in fractionation procedures to obtain at a certain pH a precipitated complex with a biological macromolecule. After centrifugation and separation from other soluble components, the precipitate can be easily dissociated again by changing the pH or salt concentration. Such a procedure is now under study as a means of fractionating the endotoxin detoxifying component of Landy.

Additional information has been obtained on the polysaccharide (P-45) prepared from Serratia marcescens by a new, large-scale method of Mr. A. Perrault reported on last year. This polysaccharide endotoxin is an acid polysaccharide complex. In earlier work with Dr. E. Merler, Dr. Mora found 80-100 negatively-charged groups with pKa = 2.3 in a 100,000 molecular weight unit. With Mr. B. G. Young, the titration of the negative polysaccharide with positively-charged macromolecules, such as polymyxin, protamine and lysozyme, has been studied. There was a neutralization observed at a pH range where the maximum increase in turbidity indicated that strong macromolecular interaction (precipitation) took place. Generally, with the positive macromolecules the tumor necrotizing activity decreased, but the pyrogenic activity remained constant. However, after interaction with lysozyme the tumor necrotizing activity slightly increased, and after prolonged incubation for one or two weeks the fever-producing activity substantially decreased. The interaction with lysozyme was accompanied with changes in the sedimentation: lower molecular weight components appeared. Some fractionation studies were carried out in the ultracentrifuge in collaboration with Dr. W. Carroll, (NIAMD). The P-45 preparation was also found to be effectively deaggregated by treatment with the anionic detergent: sodium lauryl sulfate. Sedimentation of the original P-45 in the ultracentrifuge shows two peaks, one $S^w_{20} = 4$, and another $S^w_{20} = 12$. After treatment with sodium lauryl sulfate, there appears only one peak at $S^w_{20} = 2$. However, up to now, the sodium lauryl sulfate could not be removed without causing reaggregation. The biological activity of the $S^w_{20} = 2$ component was unchanged in the presence of the sodium lauryl sulfate. This indicates that the biological activity is retained in a relatively small molecular weight unit of about 10,000 to 20,000.

These studies are of importance in understanding the relationship of biological activity of a macromolecule and the types, number and distribution of active sites on the molecule. Studies have been initiated to differentiate the enzymatic and antigenic sites on an enzymatically active protein (RNAase) by electrostatic complexing and blocking of certain limited portions of the protein surface. Other serum components interfered with the enzymatic assay when impure RNAase - antibody was added to RNAase, and purified antibody is now being prepared (method of Singer). Under study are influences on enzymatic activity of complexes with such

antibody and effects of adding negatively-charged macromolecules on enzyme activity, enzyme - antibody interaction, and in vivo development of antibodies to RNAase. Enzyme - substrate interactions are also being investigated by similar approaches.

Metabolism of the Tumor-Bearing Host:

Body Composition Studies:

Drs. J. White and F. Miller and Mrs. J. Toal (in collaboration with Drs. J. Bloch, A. Harris and N. Berlin) continue to add knowledge on the changes in body composition and physiology which occur in tumor-bearing rats. Last year, in a study designed to gain information on the increased requirement for sodium ion by rats bearing subcutaneous, progressively growing Walker carcinosarcoma 256, it was found that when the tumor was more than 10 percent of the body weight pronounced retention of sodium occurred. Analyses of carcasses and tumors have now shown that the amount of sodium found in the tumor accounted for almost all of the total sodium retained. When the concentrations of nitrogen and electrolytes in carcasses of rats bearing the tumor were expressed on a fat-free, wet weight basis, it was found that sodium, chloride and nitrogen were increased above normal values and potassium and water were decreased. Samples of muscle and bone showed no increase in sodium and chloride ion concentrations, and, although increases were observed in skin, they were not enough to account for the increases found in total carcass. From studies with undernourished mice, it appears that, with respect to carcass composition, the tumor-bearing rat in positive nitrogen balance resembles a nontumor-bearing rat in negative nitrogen balance. Analyses of tumor composition show higher concentrations of sodium and chloride and lower concentrations of potassium and nitrogen in necrotic tissue than in viable tissue.

Rats bearing the Murphy-Sturm lymphosarcoma intramuscularly were also studied. Carcasses showed changes similar to those observed with the Walker tumor-bearing rats. Total sodium retained by the Murphy-Sturm tumor-bearing rats, however, was much less than that retained by the Walker tumor-bearing rats.

In order to compare the rate of equilibration of water in a variety of tumors with water in blood and other tissues, Drs. Millar and White injected tritiated water intravenously into ether-anesthetized rats and mice bearing different primary or transplanted tumors and obtained samples of cardiac blood, tumor and other tissues at subsequent times. From radioactivity measurements on the water vacuum-distilled from the samples, equilibration values from the tissues were compared with those of cardiac blood samples. With the exception of mouse ascites

tumor, all viable tumor tissues reached equilibrium with blood in 5-10 minutes. Tumor equilibration time for the tumors were similar to those for muscle, greater than those for liver, but less than those for skin. Mouse ascites tumor equilibration times were about thirty minutes. The results suggest that the major factor in regulating water exchange in tumor tissue is the blood supply to the tumor. The findings have implications of importance to the study of the rates at which drugs enter tumor and normal tissues, and antitumor drugs will be evaluated.

Previous work by Drs. R. E. Greenfield, Jr. and V. E. Price dealt with the etiology and nature of anemia of tumor-bearing subjects. These studies demonstrated that the anemia of rats with various tumors could be quantitatively correlated with the loss of cells by hemorrhage into the area of the tumor. In the course of this work it was observed that rats with marked anemia lost weight more rapidly, became cachectic and died while the tumors were in the range of 20-30 percent of the body weight, whereas in animals without anemia the onset of weight loss and cachexia was much slower, and the tumors frequently reached 50-65 percent of the body weight. However, in further study on the relationship of the anemia to the cachectic process, it was observed that during the period of most rapid tumor growth, and despite the onset of anemia, rats bearing the Lymphosarcoma R2788 gained appreciably more weight than the normal controls although they both had almost identical food consumption.

Dr. M. Rechcigl, Jr. and Dr. Greenfield have done additional experiments designed to elucidate the nature of these findings. Carcass and tumor analyses showed that the lymphosarcoma-bearing animals had an increased percentage of water sufficient to explain their increased weight gains. An equally high degree of hydration was observed in rats bearing Hepatoma 3683 whose body weight gains were lower than those of the normal controls. The combined nitrogen content of the tumor and the carcass of both groups of cancerous rats was similar to or smaller than the body nitrogen of the control animals, while the fat content of the tumor-bearing rats was markedly reduced. In confirmation of these findings, balance studies showed nearly the same retention of nitrogen in rats bearing the Lymphosarcoma R2788 as in the controls, but there was a greater retention of water and sodium, concomitant with the increased weight gains in the tumor-bearing animals.

During the terminal stages, the lymphosarcoma-bearing rats were observed to suffer from anorexia, loss in total body weight and finally died. In this period the animals were in a negative nitrogen and potassium balance, and the water retention was reduced below the normal level while the sodium balance was not appreciably affected. The positive sodium balance together with the decrease in the potassium retention indicate the

possibility that, to a considerable extent, the terminal dehydration of the tumor-bearing animals was due to loss of intracellular fluid. In these studies, the onset of anorexia and the negative nitrogen balance was not prevented by increasing the sodium chloride intake of the animals, and indeed a decrease in survival time was observed among the tumor-bearing animals ingesting saline.

Chemically Defined Diets:

Drs. M. Winitz and S. M. Birnbaum and Mr. M. C. Otey have extended their studies with chemically defined diets which were reported on last year. Earlier studies have revealed that chemically-defined diets composed of the 10 essential L-amino acids (in levels recommended by Rose et al.), 8 nonessential L-amino acids, the requisite vitamins and salts, glucose and ethyl linoleate, when provided to rats ad libitum as 50 percent solutions in water, sufficed to meet the nutritive requirements for growth, lactation and reproduction over several generations. In the attempt to develop a diet wherein each of the essential L-amino acids was present at the minimal level consistent with optimal growth, diets were prepared which incorporated each of the essential amino acids (taken one at a time) in an amount ranging from 0 to 120 percent of that previously employed. By variation of the nonessential nitrogen level all mixtures were kept isonitrogenous but were otherwise identical. Such diets were fed to weanling male, Sprague-Dawley rats over a 10-day period, and the minimal level of each essential amino acid commensurate with optimal growth was ascertained. Minimal levels of the essential amino acids, relative to the levels in diets previously studied, were as follows: L-lysine, 60 percent; L-phenylalanie, 40 percent; L-tryptophan, 80 percent; L-isoleucine, 100 percent; L-methionine, 60 percent; L-leucine, 100 percent; L-valine, 80 percent; L-histidine, 60 percent; L-threonine, 100 percent. With these ratios as the basis, it became possible to formulate chemically-defined diets whereon weanling rats grew at a rate of over 4g. per day and with more efficient utilization of nitrogen than had been observed hitherto.

It has been known for some time that certain D-amino acids could substitute for their optical antipodes, some more effectively than others, as essential amino acids required for nutrition, presumably through successive conversion to the corresponding alpha-keto acid (catalyzed by D-amino oxidase) and, through transamination, to the L-antipode. Last year studies of this type utilizing chemically-defined diets were conducted on methionine, leucine and tryptophan. Although the conversion of D-leucine to the corresponding alpha-keto acid (catalyzed by kidney D-amino acid oxidase) proceeds at an appreciably faster rate than the analogous reaction with D-tryptophan in vitro, the degree of utilization of the latter amino acid in vivo exceeds that of the former one. A comparable situation has now been found with D-alloisoleucine, which was reported in the

literature to be completely ineffective as a replacement for L-isoleucine in the diet of the rat, but which is, at the same time, very rapidly oxidized by D-amino acid oxidase in vitro. In order to ascertain why the ability of the organism to utilize the D-antipodes of the essential amino acids varies so considerably, and why results are sometimes obtained that are in variance with those that would be expected on the basis of in vitro enzymatic behavior, experiments were set up in which groups of animals were fed chemically-defined diets wherein the L-isoleucine component was replaced, in three separate diets, with one, two and three equivalents. respectively, of D-alloisoleucine. Although no growth was observed with the first two diets, a marked growth response was noted with the third. The data therefore revealed that D-alloisoleucine could be utilized in lieu of L-isoleucine if present in sufficiently high concentration in the diet. Examination of the urine of these animals also indicated that the D-alloisoleucine is excreted at an appreciably more rapid rate than is D-leucine, D-tryptophan or D-methionine and thus provided a plausible explanation for both the ability of D-alloisoleucine to support growth when provided in the diet in relatively high concentration and for the relatively lesser degree of utilization observed with D-alloisoleucine than with the other above-mentioned D-amino acids, despite its appreciable susceptibility to D-amino acid oxidase in vitro. The data indicated that the degree of utilization of D-amino acids by an animal organism is conditioned not only by the rate at which they are enzymically converted to their L-counterparts in vivo, but also by the extent of their renal resorption.

Previous experiments in the Laboratory of Biochemistry have indicated that satisfactory nutrition could be provided for rats on the chemically-defined diet offered as 50 percent aqueous solutions. From controlled experiments conducted this past year, it has been shown that the 50 percent water soluble diet is adequate for normal growth and well-being of rats, at least for a limited period of time, in the absence of independently supplied water.

In collaboration with Dr. C. I. Jarowski (Chas. Pfizer and Co.), Dr. Winitz has begun an evaluation of the concept that the free amino acid levels in the blood plasma of a given animal species, in the fasting state, might serve as a precise indicator of the dietary amino acid requirements of that species. Studies were done to compare the growth response of rats provided with the most efficient water-soluble, chemically-defined diet hitherto devised in the Laboratory of Biochemistry, with that of rats fed comparable diets wherein the essential amino acids were present in the same relative ratios as they appeared in the plasma of the fasting rat. The data demonstrated that the former and latter diets elicited comparable growth rates, despite striking differences in their essential amino acid compositions. Thus, in the rat, the concept appears valid, and, if, after work in other species, the principle becomes established, it would be of considerable assistance in the formulation of diets.

In Vivo Perfusion Studies:

In efforts designed to find a means for growing a tumor isolated from the surrounding tissues and connected with the host only by one artery and one vein, Dr. P. Gullino (Visiting Scientist), with Mrs. F. H. Grantham, has developed a technique for growing tumors in pouches developed in kidneys (or in ovaries). A kidney was isolated from the surrounding adipose tissue with the vascular peduncle as the only connection with the host. A tumor was then transplanted into the renal parenchyma, and the kidney enveloped in a bag of paraffin was placed into the subcutaneous tissue. The tumor grew in this bag and destroyed the kidney tissue; the renal vessels remained as the only connection between the tumor and the host (rats, hamsters or mice).

In rats, renal implants grew up to the size of 25 grams (about 25 times the kidney's original weight) and ovarian implants grew up to 14 grams (about 400 times larger than the host ovary). Two transplants of the same tumor, one grown in a paraffin bag and the other grown subcutaneously, showed the same correlation between wet weight, dry weight and total nitrogen and also the same level of anaerobic glycolysis. Animals bearing the "isolated" transplant behaved in the same manner as those with subcutaneous tumors as to their change in body weight, food consumption and water intake. Liver catalase was also equally depressed.

Utilizing this technique, Dr. Gullino, with Mrs. Grantham, has measured blood flow from implanted tumors and has studied the effects of certain drugs on this blood flow. In an adult rat the rate for the ovary and oviduct was determined to be about 12 ml. per hour. The same amount of blood flows out of tumors weighing 6.0 grams or more, namely 200 times heavier than the ovary into which they were implanted. The growth of a tumor implanted in a kidney, which has a blood supply 20 times larger than the ovary, produces a decrease of the total blood flow to the degree that when the tumor has destroyed all the kidney parenchyma, the blood reaches values of the magnitude of those found in ovarian implants. Despite a difference of 20 fold in the blood supply between the ovary and the kidney, equal amounts of tumor implanted simultaneously in both organs of the same rat grew at the same rate, suggesting that a large blood supply does not increase the growth rate of rat tumors. Adrenalin reduces the blood outflow of a tumor and the removal of the coeliac ganglion has the same effect. Choline increases the blood outflow in some instances.

Collagen, which was determined in collaboration with Mr. H. Taylor by measuring the amount of hydroxyproline content of acid hydrolysates of tumors, increases with the growth of the tumor. Preliminary comparative studies of some enzymatic activities in the efferent and afferent blood of the tumor are being done.

Kinetic Studies in the Tumor-Bearing Animal:

Last year Drs. M. Rechcigl, Jr. and V. E. Price reported on a method for determining the kinetics of in vivo synthesis and destruction of the liver enzyme, catalase, by use of 3-amino-1, 2, 4-triazole. With Dr. R. Hartley, they have evaluated the validity of this method by an alternative one in which catalase synthesis was blocked by the use of allylisopropylacetamide administered intraperitoneally to rats. When synthesis was blocked, activity of the remaining catalase disappeared with a first order constant, K_d, of 0.023, corresponding to a rate of disappearance of 2.3 percent per hour, a figure almost identical to that obtained by the older method.

These methods have been employed in comparative studies with rats on a protein-free diet, on a starvation diet, or bearing a tumor. In the liver of starved animals, synthesis and destruction proceeded at essentially the same rate per gram of tissue as in normal animals for the first 5 or 6 days of starvation. The liver and total liver nitrogen is continually decreasing in size during this period, however, so that there is actually less catalase being synthesized and destroyed in the total liver. In contrast to the picture seen in starvation, animals on a protein-free diet of carbohydrate, fat, minerals, vitamins and a source of fiber showed a decreased rate of catalase synthesis per gram of tissue or per mg. of nitrogen. Although there was less catalase being synthesized, the fraction of catalase molecules being destroyed per unit time was within normal limits, thus resulting in a lower concentration of catalase within the liver. However, in the animals on the protein-free diet, the liver size and total liver nitrogen decreased only slightly.

The effects of tumors are more nearly duplicated by the protein-free diet, for in the tumor-bearing animals there is a marked fall in the catalase activity per gram of liver, and the liver is frequently somewhat enlarged. In both these states the total liver catalase is markedly reduced as it is in starvation, but it is not accompanied by the marked decrease in liver size or total liver nitrogen seen in starvation.

Enzyme Activities of the Tumor-Bearing Host:

Comparative studies of catalase activity of various hepatomas and of their effects on host liver and kidney catalase activity levels have also been made by Drs. Rechcigl and Price, in collaboration with Drs. H. P. Morris and H. Sidransky. A fairly high catalase activity was found in the 5123 hepatoma and in the primary and the transplanted ethionine-induced hepatomas of mice. Almost no activity could be demonstrated in the Dunning, Novikoff and the 3683 hepatomas of rats.

Despite the fact that fairly high levels of catalase were found in the tumor, the catalase activity of the liver and kidneys of animals bearing 5123 and ethionine-induced hepatomas was depressed to a degree comparable with the other tumor-bearing animals tested. The amount of depression was correlated with the size of the tumor and the loss of the carcass weight.

In view of the lowering of the liver catalase of normal animals following the feeding of a protein-free diet, it was of interest to find out whether a comparable decrease in the enzyme activity would occur in hepatoma 5123. The experiments have shown that a protein-free diet produces no significant lowering of catalase in the 5123 hepatoma, even though the catalase of the liver was markedly reduced.

In spite of numerous reports that a substance isolatable from tumor tissue (toxohormone) causes reduction of liver catalase activity by liberation of the substance into the blood stream with local action on the liver catalase, other interpretations of the phenomena may well deserve equal or better support. The reduction in catalase activity observed in bled animals and in those placed on protein-free diet (vide supra) suggest other mechanisms, such as a relative nutritional deficiency or an alteration of the homeostatic balance brought about by competing altered metabolic pathways. The kinetic models designed to permit the simultaneous measurement of catalase synthesis and destruction, especially if confidence in their validity can be maintained, for nonsteady states present in tumor-bearing animals as more work is done, are likely to aid in the clarification of this well-known effect on the host by a tumor.

Transplantation of a primary ethionine-induced hepatoma resulted in tumors having two different levels of catalase activity, one of which was twice that of normal liver, whereas the other was about half the normal level. Enzyme level estimations in tumors of later transplant generations of the two lines will be of interest.

Utsugi has recently reported that hypophysectomy will prevent the depression of liver catalase activity. Drs. Rechcigl and Price, with Dr. S. Wollman, however, found a marked fall in the concentration of liver catalase in hypophysectomized animals bearing transplanted Wollman thyroid tumors. In fact, the effects of the tumor growth and removal of the pituitary gland were additive. Starvation is often referred to as pseudohypophysectomy, since the effects of starvation and extirpation of the gland are similar. The similarity was also seen in experiments involving liver catalase measurements in hypophysectomized rats, since the livers were only about one-third the normal size, and the catalase activity per gram of liver tissue was at normal or slightly elevated levels.

The association of elevated serum aldolase and lactic acid dehydrogenase with certain tumors and with certain lesions of muscle has been well-documented in both human and animal subjects. Recently, Riley has reported marked elevation of serum lactic acid dehydrogenase in mice that had received injections of cell-free extracts. Drs. Greenfield and R. Berry have confirmed these findings in mice; however, rats injected with a large series of transplanted tumors had no such elevation, and cell-free extracts of tumors were also without effect in these animals. Preliminary studies suggest that the elevation of lactic acid dehydrogenase as seen in mice is not due to a viral agent since elevation was still obtained following administration of cell-free extracts irradiated with a dose sufficient to inactivate most viruses.

Deoxyribosides of the Tumor-Bearing Host:

Investigation of the deoxyribosides of rat tissue and the metabolism of these compounds during liver regeneration and growth is under way by Dr. J. Rotherham. Deoxycytidine, 5-methyldeoxycytidine, deoxyuridine, and unidentified deoxynucleotides were found in the urine of normal rats fed a casein diet. When rats are maintained for two or three weeks on a diet which contains no source of preformed deoxyribosyl compounds, these compounds are still continuously excreted in the urine, an indication that they are formed endogenously (bacterial action has not been ruled out). Studies with this special diet indicated that the level of excretion fell in hepatectomized animals on the second day after the operation. A fall in levels of excretion occurred in chow fed animals bearing the Novikoff hepatoma when the tumor was growing rapidly, but a similar fall was observed in pair-fed control animals.

Urinary Excretion Patterns of Nucleic Acid Congeners in Leukemia Patients:

Further progress continues to be made by Dr. J. Reid and Mr. W. Bell in the difficult and complex investigation of the urinary excretion patterns of nucleic acid congeners and other substances in leukemia and normal human subjects. The methods used are primarily chromatographic with employment of ultraviolet absorption spectra determinations of the eluate fractions; machine methods of data processing are being introduced. Dietary composition exerts an important effect on the normal urinary pattern. The absorption curves were changed in all chromatographic fractions when a freely selected diet was given compared with the results seen when a low-purine, low-ribose control diet was administered. Patterns seen in urines from two untreated patients with acute myeloblastic leukemia and from two untreated patients with acute lymphoblastic leukemia differ from those seen in urine from normal subjects.

Vector Analysis-Computer Techniques:

In a study designed to test the feasibility of analyzing multicomponent mixtures (by means of vector analysis-digital computer techniques applied to the ultraviolet absorption spectral curves of chromatographic eluates of ribonucleic acid (RNA) hydrolysates), Drs. Reid and A. Pratt have made considerable progress. With RNA as a model, it is intended to develop the techniques to a point where they can be used to analyze urinary excretion patterns from leukemia patients. RNA hydrolysates consisting of the four major nucleotides and three other components present in smaller amounts have been analyzed by the computational method with a precision equal to or better than that of standard methods and an accuracy about as good. When the spectra of the minor components have been determined, the accuracy will be increased. With the aid of Mr. N. Coffey, automation of the system is being accomplished, thus permitting future practical application of the approach to large numbers of samples.

Energy Expenditure Studies:

Further improvements in mathematical formulation and instrumentation utilized in direct and indirect calorimetry studies have been made by Dr. Pratt and Mr. W. White. Particular attention is given to methods because of the need for high precision in evaluating the independent measures of energy expenditures (direct and indirect) of the tumor-bearing and normal animal. From mathematical approaches developed by Dr. Pratt to the solution of the dynamic water pool determination and the determination of the body volume of an animal in the respiratory chamber, it appears possible with the use of tritium-labeled water to obtain samples of respiratory water without disturbing the animal and, if measurements can be made with sufficient accuracy, to estimate the total body water and body volume of the animal in the chamber. By use of density measurements of the dry total carcass and of bomb colorimetric measurements of the calories per gram of dry total carcass, Dr. Pratt is now able to calculate from relationships he has formulated, the nonfat and fat fractions without the necessity of chemical separation. These new approaches provide additional means for evaluating independent measures of energy expenditures, one of the important areas of investigation of metabolism of the tumor-bearing subject.

With Drs. H. Benzinger and C. Kitzinger (Naval Medical Research Institute, Bethesda, Md.), Dr. Pratt is developing techniques of clinical thermometry to ascertain body temperature-energy metabolism relationships. A basic relationship between skin temperature, intracranial temperature and energy production has been found in studies on human subjects. The methods developed should also prove useful in other types of clinical studies.

TUMORS AND THEIR PROPERTIES

Staff members of the Institute maintain about 200 transplantable neoplasms. This very valuable resource includes many tumors of special characteristics and is a vital part of the armamentarium of cancer investigators, not only within the Institute, but throughout the world.

As part of a long-term study of the effect of age on rats of six of the NIH inbred strains, Dr. K. Snell reports differences among them as to the most frequent sites of spontaneous tumor formation: Osborne - Mendel, adrenal cortex; Buffalo, anterior pituitary gland and adrenal cortex; AXC, testis and anterior pituitary gland; Fischer, testis; M520, adrenal medulla; and Wistar, anterior pituitary gland and mammary gland.

Acetic acid-soluble collagen in hamsters, which is present in lower amounts in back skin as compared with abdominal skin, has been found by Dr. W. Banfield to decrease in skin with increasing age except for a rise in the amount from abdominal skin between the ages of 28 and 42 days. In the process of maintaining old hamsters while developing this useful tool for study of collagen metabolism, Dr. Banfield has also established 16 transplantable hamster tumors (among which are 2 melanomas, 1 adrenal cortical tumor, 1 myxoma, 1 epidermoid carcinoma, 1 adenocarcinoma of the bowel and 2 reticulum cell sarcomas).

Dr. M. Stanton and colleagues have initiated a study on the induction and natural history of conjunctival carcinoma in Hereford cattle. Histology and electron microscopic examinations of biopsy material are under way from a herd of cattle in Eastern Maryland that has an incidence of 90 percent. The possibility of an insect-borne virus as the etiological agent will be investigated, and virological studies are in progress.

Miss D. Uphoff is attempting to establish a line of mice, C57BL/10D, that develop multiple polyposis of the colon. Although polyps are occasionally found, malignancies have not been observed.

The biologic behavior of the adrenal cortical carcinoma 494 (i.e., rats bearing the tumor develop polyuria, polydipsia, degenerative changes in the kidney, and atrophy of adrenal glands, sex organs, lymphatic tissue, and pituitary glands) has remained the same through 15 transplant generations in studies by Dr. Snell. In a collaborative study, Dr. Johnson (NIAMD) has found that desoxycorticosterone is the major steroid elaborated by the tumor, even though this steroid is absent or nearly so in the adrenal glands of Osborne-Mendel and other strains of rats.

In an attempt to learn of differences between steroid secretions by another adrenocortical carcinoma and normal adrenal cortical tissue of the rat and to obtain additional understanding of metabolic pathways related to tumor - normal tissue comparisons, Drs. A. Mulay and E. Price, Jr. have found that rats bearing the tumor show atrophy of the zona fasciculata and zona reticularis of the adrenal crotex, a sharp reduction in urinary sodium excretion (both in intact and adrenalectomized rats), increased potassium excretion, hypernatremia, and hypokalemia. Since studies on stress in tumor-bearing rats demonstrated that the tumor could protect the animals against intoxication produced by intraperitoneal potassium chloride injection, the tumor apparently elaborates mineralcorticoid secretions. Preliminary findings suggest no production of glucocorticoid secretion by the tumor.

Dr. S. Wollman, as part of a continuing study on properties of transplantable rat thyroid tumors, has found that one tumor line can maintain a concentration of radioiodine elevated above that of blood and nearly as high as that in normal thyroid glands, although this tumor cannot form appreciable protein-bound I^{131}. Impairment in transport of radioiodide from blood to tumor has also been observed. Dr. J. Tjio (NIAMD) has examined the chromosome complement of three lines of thyroid tumors maintained by Dr. Wollman and found that two anaplastic lines had deletions of 4 and 7 chromosomes, respectively, whereas a functional line had a deletion of only 1 chromosome.

A number of spontaneous and induced mouse tumors have been injected intravenously by Drs. R. Malmgren and E. Chu, and comparisons were made of the nuclear DNA content between the initial tumor and the developing metastases. There is a tendency for the metastatic tumor cells to have a higher nuclear DNA than the primary tumor from which they are derived; however, it appears that the high ploidy cells are not the only ones that are capable of establishing metastatic foci.

Mouse tumors and normal tissues and extracts prepared therefrom when injected into mice were observed by Dr. Malmgren to increase the mitotic activity of liver under certain conditions. The factor appears to be heat stable and can be stored in vacuo. Heated normal tissue produces an increase in mitotic activity, but unheated normal tissue appears to contain an inhibitor, since it can block the effect of tumor tissue.

With Dr. E. McLaughlin, Dr. Malmgren has observed an increase in liver mitotic activity in mice and rats that received serum from cancer patients. The effect was not seen when serum from normal human subjects was given. Using tritium-labeled thymidine and isolating DNA from the livers at 72 hours, Drs. Malmgren and McLaughlin observed an increase in new DNA in the livers of partially hepatectomized rats treated with

serum from cancer patients compared with rats treated with normal human serum.

Dr. Malmgren found no significant difference between the various cells studied from carcinoma in situ and invasive cancer patients with techniques of microspectrophotometry, interference microscopy, and planimetry while he was working in Professor T. Caspersson's Laboratory in Stockholm for a three months' visit.

In continuing investigations on cytochemical changes in intact living individual cells, Drs. G. Williams and A. Peacock have made further improvements in instrumentation used for ultraviolet-television microscopy and time-lapse cinematography. By use of this system, intact cell functioning can be studied with brief exposure of low-level UV radiation, and they have completed a study of a series of tetrazolium salts utilized to investigate the reaction rates of an enzyme system catalyzing the reduction of tetrazolium to formazan and to obtain information on the cellular localization of the reaction. In liver cells, progressive reduction occurs in the vicinity of mitochondria without appearance of formazan outside the cell or inside near the cell surface. Tetrazolium appears to be freely permeable, and all but 20 percent bound to cellular protein can be easily removed with a single washing of the cells. Improvements in instrumentation include new lenses that permit excellent resolution with UV light at 265 mμ in enlargements at a magnification of about 30,000 diameters and a new electronic shutter with precise opening times from 5 to 75 milliseconds, or 10 millisecond intervals at 5, 10, 15 or 30 cycles or 2 or 3 minute cycles. Exposure of the cells to UV light with this TV arrangement is about 1/100th that required to photograph the activity directly on film. With 10 millisecond exposures at intervals of 15 seconds, human leukocytes tolerate UV light at 265 mμ for 15 to 30 minutes before showing structural and dynamic changes of damage.

Plasma Cell Tumors:

Dr. M. Potter has made additional progress in his investigations on plasma cell neoplasms. He has found that BALB/c mice given single intraperitoneal injections of adjuvant-staphylococcus mixtures, develop plasma cell neoplasms in the lipogranulomatous tissue that formed in all mice receiving the mixture. Thirty-one plasma cell tumors have been found thus far, none developing prior to 5 months after injection. Because of the complexity of composition of the adjuvant mixture, studies are under way to determine if single components and other materials will also produce these neoplasms. To date, 4 plasma cell neoplasms have developed following the administration of paraffin oil (Bayol F) alone to 40 mice. No plasma cell tumors have appeared to date in similar studies in three other strains of mice.

Growing transplants of plasma cell tumors produce quantities of serum myeloma globulin in new recipients. Specific myeloma globulins are produced by different tumors. When a tumor is established in transplant, the specific myeloma globulin production is constant for that tumor and remains qualitatively the same throughout the transplant history. Studies on different protein-producing tumor lines derived from a single host would be of interest. One case so far has been found: the primary host of Adj-PC6 gave rise to two different, stable transplant lines, Adj-PC6A and Adj-PC6C. One difference in the behavior of these two cell lines results in precipitation of the myeloma globulins when whole serum of tumor-bearing animals is diluted in low ionic strength media in the case of the former, but not the latter cell line. Of the 24 different plasma cell neoplasms (20 are of strain BALB/c origin), two additional cell lines give this precipitation phenomenon; in such cases isolation of the globulins is simplified.

The primary peritoneal plasma cell neoplasm appears to arise multifocally, for the reactive mesentery contains numerous nodules of seemingly incipient plasma cell tumors. Dr. Potter is using an ingenious method to study the mechanisms of their development by transplantation of wedges of mesentery containing separated nodules into recipient mice. By this method he hopes to localize the time and site of origin of the carcinogenic event and to obtain discrete tumors from which lines can be established for additional immunological and chemical characterization of the tumor products.

Several immunochemical and cytochemical studies are under way with Dr. E. L. Kuff. The microsomes contain material antigenically related to the serum protein myeloma globulins. Ribonucleoprotein (RNP) particles prepared from microsomes by deoxycholate treatment also carry the antigenic groups of the myeloma proteins. Similar antigenic determinants are found on normal immunoglobulins such as gamma-2-globulin (SI) and a certain beta-globulin (SII). Three antimicrosome antibodies have been studied:

Tumor source	Precipitates formed with normal serum
MPC 1	SII
MPC 2	SI, SII
Adj-PC 5	SI.

Myeloma globulins are related antigenically to SI or SII, or very rarely to both. The normal SI and SII proteins contain different antigenic determinants. Myeloma globulins may contain different antigenic determinants, or may lack them. Characterization of the myeloma

globulins of BALB/c mice will be done, and the degree of genetic control obtainable in such a study will be far superior to that possible with myeloma globulins sampled at random from heterogeneous populations.

Dr. M. Landy and Dr. Potter have sought to determine whether the abnormally high levels of beta and gamma globulins in the sera of mice bearing plasma cell tumors (induced with paraffin oil and bacteria) represented antibody or disoriented protein. The serum of mice normally contains, in low titer, a broad spectrum of individually specific antibodies to Gram-negative bacterial species. As far as can be determined, these antibodies are the result of antigenic stimuli supplied by continuous contact of those animals with Gram-negative bacteria. It was reasoned that in the presence of this persistent, multiple, natural antigenic stimulus, the greatly increased synthesis of serum globulins, usually associated with antibodies, might be reflected in markedly increased levels of antibody to these bacterial species. Employing the sensitive and objective bactericidal assay for antibodies to Salmonella, it was found that sera of mice with these plasma cell neoplasms contained no greater amount of Salmonella antibody than that present in normal mice. Since the increased level of globulins did not reflect enhanced antibody response to bacteria, the function, if any, of globulins is still obscure.

In order to learn more of the origin of plasma cell tumors, Dr. R. MacCardle is studying the origin and ontogenetic development of plasma cells in mice and attempting to learn if these cells can be formed earlier under artificial stimulation. In BALB/c mice, normal plasma cells first appear in the lymphatic tissue of the intestinal mesentary at about ten days after birth. These young cells possess Golgi apparatus considerably smaller than those in adult cells.

In an investigation also concerned with the nature and origin of plasma-cell tumors, Dr. MacCardle, with Dr. Potter, has noted three types of Golgi apparatus; the type of Golgi apparatus does not appear to be related to the electrophoretic mobility of globulin produced, but rather to the rate of production of the protein. The cells of some of the plasma cell tumors appear to derive from undifferentiated mesenchymal cells around blood vascular capillaries and adipose cells of the intestinal mesenteries and parietal peritoneum. After transplantation into hosts, some grow in fascicles in the interstices of a blood vascular network of the transplant. Normal plasma cells develop in similar fashion following intraperitoneal injection of silica or mineral oil.

Using microincineration and Brachet's pyronin-methylene green-ribonuclease techniques, Drs. MacCardle and Potter have found that gammaglobulin secreting plasma cell tumors are generally rich in

cytoplasmic RNA and in calcium and magnesium, whereas those secreting alpha- and beta-globulin are poor in cytoplasmic RNA and rich in intranuclear RNA. The calcium and magnesium content of the latter varies. The nucleolus of the gamma-globulin tumors is large and rich in silica.

In collaboration with Drs. Potter and Ruth Merwin, Dr. A. J. Dalton has examined by means of electron microscopy 17 primary and transplanted plasma cell tumors and controls consisting of normal stimulated and unstimulated plasma cells or fibrosarcomas. Maintenance of ultrastructural features characteristic of the plasma cells is related to retention of function (determined by the pattern of specific myeloma globulins elaborated by a particular cell type). The evidence obtained also suggests that particles present in the cytoplasm of cells of all the plasma cell neoplasms examined (but not in normal plasma cells nor in the cells of other neoplasms) are not the morphologic representation of an oncogenic agent responsible for the development of the tumors.

Dr. H. Kobayashi (Visiting Scientist) with Drs. Thelma Dunn and Potter, has demonstrated both osteolytic and osteoplastic reactions by plasma cell neoplasms using techniques involving intravenous injection of the ascites form of tumor (growth within the bone marrow resulted), transplantation of solid tumor tissue into marrow cavities and onto peritoneal surfaces, the use of papain-cleared skeletons, and examination of histologic sections.

Diffusion Chamber Studies:

The occurrence of several plasma cell tumors and sarcomas in mice following the placement in the peritoneal cavity of millipore filter diffusion chambers was reported previously by Dr. Ruth Merwin. In further work with Dr. Thelma Dunn, she has found that both tumor types are induced more frequently in the BALB/c than in the C3H strain and that the presence of tissue inside the chamber, stilbestrol infection, and anyone of the chamber parts (membrane, lucite or adhesive) do not appear to be essential to induction of the plasma cell tumors. BALB/c mice react by producing intraperitoneal plasma cell neoplasms which are transplantable and have characteristic protein electrophoretic patterns resembling those of human multiple myeloma. The C3H mouse, however, has not produced these neoplasms, but severe fibrosis and occasional ossification occur.

Dr. Emma Shelton has continued her study of the behavior of normal and malignant cells grown in double diffusion chambers placed in the peritoneal cavities of mice. Fluids, but not cells, may pass freely through the membranes of the chambers. Growing in the chambers,

lymphocytes that were procured from the thoracic duct and lymph nodes behaved differently from those procured from the peritoneal cavity, indicating different potentialities for survival, growth and differentiation. The latter formed organized fibroblastic sheets and became a minor component in the cell population; the former produced few sheets and persisted as major components in the population. Peritoneal lymphocytes grown in the chambers on different physical forms of substrate membranes varied in their capacity to form organized tissue and failed to undergo the transformations observed when grown on Millipore substrate membranes, except when they grew in the fibrous coagulum that sometimes formed. No differences were seen when either basic or acid ion-exchange resins were included in the chambers. Preliminary findings with chemical techniques show an increase in mucopolysaccharide correlated with connective tissue growth. Immunological techniques demonstrated passage of antibody into the chambers. These different responses of cells obtained from different body sites may be of importance in determining the factors responsible for establishment or lack of establishment of metastatic lesions in different tissue sites in the body.

In collaboration with Dr. Virginia J. Evans, Dr. Shelton has found that mouse connective tissue grown for at least a year in diffusion chambers (through 10 to 12 chamber transplant generations) did not acquire malignant properties when evaluated by the technique of intraocular transplantation.

Dr. R. T. Bradley (last year an Anna Fuller Fund Fellow) and Dr. L. Law are continuing studies with a technique that allows one to perfuse substances into the extracellular fluid environments of a designated piece of tissue or population of cells within normal unrestrained experimental animals for periods up to several weeks. One study has utilized this procedure to perfuse substances continuously into intraperitoneally implanted Millipore filter diffusion chambers containing P-388 or L1210 cells. When such chambers were perfused with Eagle's basal medium (with pyruvate, serine and glutamine) for 7 days at the rate of 1 ml. per day, the approximate generation time was 12 hours, compared with 24 to 32 hours in control nonperfused chambers. Other perfused media gave poorer growth rates, and the effects of various substances are under study.

Tissue Culture Studies:

The careful technique sustained over the years in the Tissue Culture Section has permitted the cell cultures to be carried aseptically, with an absolute minimal and usually no use of antibiotics. Recent studies have shown that all cultures carried in the Section are apparently free from pleuropneumonia and diphtheroid organisms which have complicated the interpretation and evaluation of nutritional, biochemical and other biologic data from laboratories relying on antibiotics. The Section is believed to

be the only large-scale, long-term tissue culture laboratory whose experimental results have not been complicated by such contaminations.

Comparative studies of normal and malignant cells can be facilitated if additional techniques are developed that will enable cell strains, already adapted to static cultures in chemically-defined media, to grow luxuriantly in fluid suspension cultures without addition of serum or other ill-defined components. Agitated culture techniques produce stress on the cells grown in fluid suspension which varies considerably with the conditions employed. Messrs. J. C. Bryant and E. L. Schilling and Drs. V. J. Evans and W. Earle are investigating a number of factors that appear important in growing cells in agitated suspension, such as the shape and size of the containers, the type of surface of the containers, the rate of agitation and the presence or absence of certain components in the medium, particularly polymers of known composition that might play a beneficial role similar to that of serum. Last year it was reported that addition of methylcellulose to the chemically-defined medium NCTC 109 produced a protective effect on cells grown in agitated culture. Higher concentrations of the polymer than used last year have led to increased proliferation rates of monkey kidney cells. Additions of spermine or much higher concentrations of folic acid than ordinarily employed did not give improved results (from reports in the literature, spermine has been shown to influence permeability of bacterial cell walls by preventing osmoticlysis and high concentrations of folic acid appear to be required for certain kidney cell lines grown in static culture). In roller tubes, different rates of tube rotation (from 19 to 2400 rph.) have been investigated; 1200 and 600 rph. gave higher proliferation rates than those at 2400 rph. or 300 rph. or less.

Further improvements have been made in techniques utilized in growth of mammalian cells in rapidly agitated fluid suspension cultures grown in shaker flasks by Messrs. Bryant and Schilling and Dr. Earle. By reduction of the size of the individual culture flasks, the entire agitation assembly can be placed within the carefully controlled temperature room, and manipulation of the cultures is greatly facilitated since the fluids can now be changed without transfer of the cells from the flasks. This new procedure now allows rapid proliferation of monkey kidney epithelial cells in a protein-free, chemically-defined medium for the first time.

Dr. Evans is attempting to adapt freshly isolated cells of monkey kidney, mouse kidney and mouse fibroblasts to grow in a protein-free, chemically-defined medium NCTC 109. Maintenance of cells and some indication of growth has taken place, but it is too early to consider that cell lines have become established. Establishment of such lines in protein-free medium would be of importance to virology studies since the problems

encountered with serum-containing media could be avoided. Furthermore, pleuropneumonia-like organisms would be excluded since the medium does not propagate these organisms.

In collaboration with members of the staff at the Navy Tissue Bank (National Naval Medical Center, Bethesda), Dr. Evans has also successfully cultured human skin epithelial cells in protein-free medium NCTC 109 in both static and agitated cultures.

Dr. Earle and Mr. W. T. McQuilkin are attempting to establish the cinemicrographic techniques to permit accurate characterization of cells in culture, including growth rates, cytokinetic periods, intermitotic intervals, and migration rates. Considerable variability of behavior among cells of pure line populations makes progress toward standardization of technique slow. Even with preliminary findings, however, it is evident that a cell line (NCTC 929-strain L) returned to serum-containing medium after an extended sojourn in a protein-free, chemically-defined medium (NCTC-109) reverts to a state exhibiting characteristics seen in the original strain, but not seen while grown in the protein-free medium. Thus, whatever the phenomena of adaptation to the chemically-defined medium might be, the observed changes are not necessarily permanent. It may be recalled that last year's report indicated the changes seen when cells of this clone line were adapted to growth in a protein-free medium: the average generation time was prolonged; the migration rate on the glass surface was greatly increased; the cells became less compact, refractile and granular and rounded up only in prophase.

Transformation Studies:

During the past few years Dr. Katherine K. Sanford and her associates in the Tissue Culture Section have studied many types of transformation -- neoplastic, enzymatic, morphologic, and chromosomal -- occurring within clones of mammalian tissue cells grown in long-term culture. With the identification of several biochemical markers, such as those determined by Dr. B. B. Westfall, and a number of nutritional variants, the tools may now be at hand to learn of the mechanisms involved in these transformations, to attempt to control and induce them, and to test for the transfer of genetic materials between cells. Recently Dr. Sanford and her associates have reported additional studies on the development in vitro of hereditary differences in morphology and further alterations in arginase and beta-glucuronidase activities within a single-cell clone of mouse tumor cells. By means of clonal analysis additional hereditary alterations in morphology, level of enzyme activity, tumor-producing capacity, and chromosomal constitution have been determined among clonal derivatives. Last year an initial attempt to identify "hybridization" of cells produced by transfer of

genetic materials between cells was not successful, but encouragement for new attempts now comes from work by Barski, Sorieul and Cornefert, who, using clones obtained from Dr. Sanford, have demonstrated "hybridization" of cells as evidenced by chromosome markers (see also the report below in the section on Nucleic Acids by Drs. Law, Bradley and Roosa).

Dr. Virginia J. Evans has studied three series of fresh C3H embryo explants cultured in medium NCTC 109 and 10 percent horse serum to determine the time and incidence of transformation to malignancy as determined by intraocular injection following in vitro growth periods of different lengths. Tumors occurred after the cells were in vitro only 176 days, 147 days and 138 days, respectively, for the three series. Growth acceleration of the cultures occurred some 4 to 8 weeks prior to the time the transformation took place in vitro. The tumors produced in the eye were fibrosarcomas, with latent periods ranging from 17 to 125 days. Exploratory chromosome counts indicated that there were many heteroploid cells in the populations (mostly hypo or hypotriploid). Such information pinpoints rather sharply the time at which the investigator must look for leads in understanding the transformation and provides a fairly clear-cut calendar for other studies requiring knowledge regarding the state of the cells in culture.

Similar studies with medium NCTC 109 without added serum have been done with implantation intraocularly after 26 to 211 days in vitro. Resorption of all implants has occurred to date. It should be pointed out that under these conditions, the rate of growth in vitro has been found to be slow, with death occurring and waves of new cells growing out.

Fibroblasts obtained by implanting small pieces of epigastric fat pads in double millipore filter chambers (porosity HA) which were then placed in the peritoneal cavities of mice and grown through 12 successive chamber transplant generations gave good growth in vitro. Implants into the eye survive, but do not proliferate.

Dr. Sanford also continues to investigate several variables of cells grown in culture beyond short term periods through study of effects of vitamins varied, singly or in combination, in their concentrations. Medium NCTC 109, a protein-free, chemically-defined medium that supports long-term proliferation of cells grown in culture and was developed a few years ago, is extremely complex in composition. Although a simplified medium, NCTC 118, is an improvement, it nevertheless contains 18 vitamins. As reported last year, when each of the 18 vitamins was individually deleted from the medium, 5 vitamins (calcium pantothenate, choline chloride, niacinamide or niacin, thiamin and riboflavin) were identified as essential for cell survival. Vitamins not apparently required for cell survival, but

which increased rates of cell proliferation were folic acid and pyridoxal (or pyridoxine).

During the past year, two new media were evaluated, and the interrelationships of vitamin B_{12}, folic acid and thymidine were studied in their effects on cell proliferation and growth. Medium NCTC 119, containing only 7 vitamins, failed to support cell survival for more than two or three weeks. Additional studies with five other vitamins showed an importance of biotin which could not be demonstrated in the presence of all 18 vitamins. Investigations with medium NCTC 120, which contains biotin, showed that the cells, though proliferating as rapidly as the controls in medium NCTC 118, were fragile and could not withstand the stirring used to set up quantitative experiments. When vitamin B_{12}, folic acid or thymidine were deleted individually from the medium, no detectable effect on cell survival or proliferation was noted. When all three were deleted, however, growth was poor, and enlarged cells with abnormal nuclei resulted. In the absence of folic acid and B_{12}, some reduction in growth was noted. A more marked reduction in growth occurred in the absence of thymidine and folic acid. In the latter medium containing only B_{12}, cells increased in nuclear mass and cell numbers decreased to a critical point. Although a few cells appeared to adapt to the medium, growth lasted only 4 to 6 weeks.

One nutritional variant which does not require pyridoxine or pyridoxal was isolated, but the cells are exceedingly fragile.

Immunological Aspects:

Utilizing red cell agglutination techniques, Dr. M. A. Fink and Dr. Evans found that in guinea pigs antibodies were produced which were specifically reactive with only homologous erythrocytes when cultured monkey or human cells carried in culture for long periods of time were administered. Surprisingly, however, neither fresh monkey nor fresh human cells incited the production of antibody reactive with homologous erythrocytes. Tests for delayed hypersensitivity revealed that both the tissue cultured and fresh tissues contained common antigens. In a comparison of fresh vs. cultured human epithelium, it was noted that pigs injected with fresh tissue reacted to human serum, while those injected with cultured tissue did not. In tests for immediate hypersensitivity (Schultz-Dale technique) there was again evident a marked diminution or absence of reactivity in the uteri from pigs injected with tissue cultured cells, but not in those from pigs injected with fresh tissue, when brought in contact with homologous serum.

These changes in antigenic characteristics seen in cells grown in culture suggest the possibility of loss of surface antigen with an uncovering

or increase in availability or potency of the antigen responsible for hemagglutinating antibodies. Should histocompatibility antigens be involved, special culture techniques might permit growth of cells containing viruses from one species and subsequent transfer to experimental animals of a different species -- another approach of importance to the study of human tumors. The above findings also confirm the usefulness of the Brand hemagglutination technique in determining the species of origin of cultured cells, a matter of considerable importance in establishing and operating tissue banks.

Enzymology:

Dr. D. B. McN. Scott (University of Pennsylvania) and Dr. Sanford have evaluated certain oxidative enzymes involved in glucose metabolism in a cloned cell line that produces tumors rapidly upon implantation in mice and in three cloned cell lines that produce tumors slowly if at all upon implantation in mice. The former showed nearly three times the hexokinase activity, four times the glucose-6-phosphate dehydrogenase activity, and five times the phosphogluconate dehydrogenase activity compared with the latter.

Nine long-term mouse cell culture strains and two human ones were all found by Dr. Westfall to produce alpha-keto-glutaric and pyruvic acids, an indication that all of them possess active transaminating enzymes needed for the formation of these keto-acids, or, in the case of pyruvate, that it was formed from glucose. On the other hand, catalase activity in twelve long-term mammalian tissue cell strains, including three derived from mouse liver epithelial tissue, was found to be very low. These findings supply additional evidence to that previously obtained by Dr. Westfall which indicates no necessary parallelism among the various enzyme systems in cells grown for long periods in culture. Some enzyme systems not necessary for cell maintenance disappear; others remain active and may be necessary for cell survival growth.

Tumors arising from injection into mice of some of the mouse cells also had low catalase activity. Hepatomas arising from injection of the hepatoma strain of mouse cells likewise had low activity. Furthermore, the liver catalase of the hosts bearing these hepatomas was not depressed.

Studies on Proteins and Nucleic Acids:

The great importance of proteins and nucleic acids in the control of functions of normal and abnormal cells and of whole organisms is attested by the vast amount of work, in many cases by leading scientists, on the subject. Like other members of the staff of the National Institutes of Health, investigators in the National Cancer Institute are making significant

contributions to the knowledge of these important materials. The fundamental studies on proteins and nucleic acids provide a base upon which further understanding of disease processes, including cancer, are built. Contributions to the separation and characterization of complex protein mixtures made by Drs. H. A. Sober and E. A. Peterson have had far-reaching influence on research conducted on the proteins and nucleic acids.

Drs. Peterson and Sober have prepared two new cellulose ion exchangers (designated ECA-cellulose and ECTHAM-cellulose, respectively) for use in the chromatography of substances that are too tightly bound to the previously available adsorbents. Both possess basic groups that are weaker than those of DEAE-cellulose or ECTEOLA-cellulose. They have been used successfully in the chromatography of ribosomes and soluble ribonucleoprotein. New elution gradients and new buffer species have been evaluated. Of special interest is tris-succinate buffer since it gives much sharper peaks than phosphate buffer in the elution of serum albumin and, in studies on trace metals, is completely combustible in the carbon arc used for metal analyses.

Sephadex is becoming increasingly useful as a dialysis replacement; furthermore, the combination of Sephadex with the cellulose ion exchangers seems like a profitable avenue of approach since a multitude of stages of continuous salt displacement can be obtained by such a mixture.

Protein Characterization:

Drs. Peterson and Sober, utilizing gradient and stepwise elution techniques in chromatographic fractionation on cellulose ion exchangers, have determined additional properties of serum fractions.

Chromatography of crystalline bovine plasma albumin (BPA) on DEAE-cellulose with a suitable gradient yields a skewed peak, suggesting heterogeneity. Treatment of the BPA with cetyltrimethylammonium bromide (CTAB) before chromatography preferentially converts the earlier, major portion of the peak into complexes having little or no affinity for the adsorbent. As the major component is thus diminished, the residual, presumably unaltered, albumin can be seen to comprise at least 4 components. Unlike the CTAB complex of human serum mercaptalbumin, that formed with BPA is largely disassociated by dialysis.

Chromatography of an old sample of lyophilized Red Cross human Fraction V gave two approximately equal peaks. The first was pure monomer; the second was 70 percent dimer and trimer. Fresh Red Cross Fraction V, on the other hand, appeared as a single, highly skewed peak containing little or no dimer.

Esterase activity of highly purified albumin, which was first demonstrated by Kramer, may represent a true metabolic function of the albumin, or it may be a fortuitous consequence of the structure of the molecule. All of the chromatographic fractions of BPA showed equal esterase activity, indicating that the activity is not attributable to an enzyme impurity. The addition of CTAB caused a 3-fold increase in esterase activity, although the same concentration of this compound had no effect on the substrate in the absence of albumin.

Progress on the isolation and characterization of the metal proteins of serum has remained slow because of difficulties in insuring the absence of contaminating metals in the solutions and equipment used. Use of plastic materials and the preparation of metal-free reagents should allow greater progress during the coming year.

Fractionation of the antihemophilic factor (Factor VIII, AHF) is progressing very satisfactorily through collaboration with Drs. Martinez-Canaveri and Casillas of Argentina. AHF has been purified with excellent recovery and the ensuing lability of the activity has been avoided in part by the addition of glucose. Although still in the preliminary stage, it also seems possible to adsorb the AHF directly on DEAE-cellulose from undialyzed and undiluted plasma from which interfering materials have been removed by prior aluminum hydroxide adsorption. This simple procedure shows considerable promise of easily providing AHF preparations that would be suitable for clinical use. Cooperative studies with the Protein Foundation are being instituted.

Dr. R. W. Hartley, Jr., working with Drs. Sober and Peterson, has studied crystalline bovine plasma albumin and a number of its derivatives by chromatographic fractionation. In the high degree of resolution obtained, the most striking result has been the separation of mercaptalbumin and nonmercaptalbumin. Subsequent experiments indicated that nonmercaptalbumin, after reduction with mercaptoethanol, behaved chromatographically as mercaptalbumin. This would indicate that some of the chromatographic differences observed are related to the presence or absence of a free SH group. Studies are under way to determine the position of this group in the albumin molecule. Albumin dimers have been found to be heterogeneous chromatographically as well as with respect to reduction by mercaptoethanol to the monomer. Dimers can be produced from the albumin monomer even in the absence of free SH groups and appear to be S-S dimers in that they are reducible by mercaptoethanol. Such studies are of importance in learning more of the physical and chemical forces involved in maintaining the native configuration and activity of many proteins.

Protein Synthesis Studies:

Dr. E. L. Kuff continues his investigation of the cytochemical organization of normal and malignant cells, with particular reference to the problem of protein synthesis and replication of intracellular components. He has used as a particularly valuable source of cells the plasma cell tumors, which have been shown by Drs. M. Potter and J. Fahey to produce and secrete into the circulating blood, large quantities of myeloma proteins while simultaneously maintaining a high rate of mitosis. Electron microscopy by Dr. A. J. Dalton has revealed the presence of an extensive endoplasmic reticulum and highly developed Golgi systems within the cells. Ribonucleoprotein (RNP) particles were extremely numerous, both in association with the neoplasmic reticulum and as apparently free entities in the cell sap. Fractionation of plasma cell tumor homogenates by standard methods of differential centrifugation gave two subfractions of the entire microsome fraction: one made up primarily of free RNP particles, and the other composed of fragments of the endoplasmic reticulum (membranes) with associated RNP particles (vide supra the collaborative work with Dr. M. Potter on the antigenic properties of these fractions).

Virus-like particles, regularly seen in neoplastic mouse plasma cells localized exclusively within the endoplasmic reticulum, have been reported by Dr. Dalton. Through the use of the nonionic detergent, Tritonx100, it has been possible for Dr. Kuff to disrupt the membrane component of the isolated tumor microsomes in such a way as to liberate the virus-like particles in morphologically unaltered form. Evaluation of the biological activity of these preparations is under way with Drs. Dalton and Law.

Through the use of a density gradient fractionation method, Dr. Kuff has been able to isolate Golgi membranes from a mouse plasma cell tumor. With Dr. R. F. Ziegel, it has been shown that the tumor Golgi fractions resemble those previously obtained from epididymus, both in their morphology and in their high content of lipids. The rate of incorporation of P^{32} into the phospholipids of isolated Golgi fractions of epididymus has also been compared with the corresponding rates for other intracellular fractions. For the relatively long incorporation periods thus far studied (one to three hours), there was little difference between the Golgi and other cytoplasmic fractions.

These studies, particularly the immunochemical studies of plasma cell tumors with Dr. Potter mentioned above, should prove to be very valuable in investigating the relationships between the synthesis of specific secretory proteins and that of the actual cellular proteins themselves. Especially important will be the studies on the microsomal RNP particles, since they may represent the sites of synthesis of these proteins. If artificial redistribution of protein during the preparation of fractions can

be ruled out or eliminated, the question can then be investigated whether the myeloma globulins are associated with all of the tumor RNP particles or only with those attached to the endoplasmic reticulum.

Drs. Peterson and Kuff have adsorbed rat liver microsomes isolated by differential centrifugation on a cellulose ion exchanger, allowing glycogen particles and residual protein to pass through unadsorbed. Because microsomes exhibit an unusual tendency to establish additional bonds with the adsorber, difficulties were encountered in the chromatography of these cytoplasmic fragments. However, with modification of techniques, clean preparations of particles having a sedimentation constant of 77S were obtained. Soluble ribonucleoprotein can also be obtained by chromatography of a clear, particle-free supernatant rat liver homogenate. Phenol extraction of the ribonucleoprotein yielded protein and an "S-RNA." The latter, upon re-chromatography, behaved identically with the original ribonucleoprotein. Further studies are under way to elucidate the reasons for the identical chromatographic behavior and to determine the completeness of the removal of protein. These techniques promise to permit separation of these important cell particulates under milder conditions than any yet reported.

Dr. M. Rabinovitz, as part of continuing attempts to introduce "biochemical lesions" within the protein synthetic pathway in normal and tumor tissues, has synthesized two antimetabolites, 4-thiamethione (antimetabolite of methionine) and S-carbamylcysteine (antimetabolite of glutamine), and has studied their inhibition of the incorporation of labeled amino acids into protein. The former inhibits incorporation of methionine, leucine, valine, lysine, and phenylalanine, and methionine does not prevent the inhibition. Glucose both prevented and reversed the inhibition, suggesting an interference with the oxidative energy supply supporting protein synthesis. The latter inhibited the incorporation of glutamine and other amino acids into protein. The inhibition of glutamine incorporation, which was not reversed by glutamine, was greater percentage-wise in the presence of higher concentrations of glutamine, and this inhibition was shown to be due to interference with the general stimulation of amino acid incorporation known to be produced by glutamine. Dr. Rabinovitz has also found that the antibiotic, puromycin, can inhibit the incorporation of amino acids into ribosomal protein, but not into soluble protein of cells.

Amino Acid and Peptide Synthesis:

The preparation of a series of alpha-amino-beta hydroxy acids (in a single step reaction which involved the treatment of an alkaline solution of an amino acid with an aldehyde, in the presence of catalytic amounts of cupric ion) was reported last year by Drs. T. T. Otani, N. Izumiya, S. M. Birnbaum, and M. Winitz. Additional hydroxyamino acids have now been

synthesized, as well as some acyl derivatives, and resolution of alpha-methyl-DL-serine and alpha-ethyl-DL-serine were attempted. Unexpectedly, the N-chloroacetyl derivative of the former compound could be resolved only partially by the usual enzymatic procedure since the hydrolysis continued on beyond the customary 50 percent point to reach 100 percent hydrolysis, indicating that the enzyme could not discriminate between the assymetry of both optical isomers of the molecule (i.e., the hydroxymethyl and methyl groups were not sufficiently dissimilar). Partial resolution could be demonstrated, however, if the reaction was stopped slightly beyond 50 percent hydrolysis. The resolution of alpha-ethyl-serine was accomplished in the usual manner.

Growth inhibition studies with E. coli were undertaken by Dr. Otani with several of the synthesized compounds. Inhibition was observed to be most effective with beta-hydroxy-DL-aspartic acid, beta-hydroxy-DL-leucine, and L-O-methylthreonine.

Dr. Winitz has successfully synthesized and isolated the epimeric mixture of delta-hydroxylysine in about 50 percent over-all yield. Separation of the diastereomers was achieved by fractional crystallization of their alpha-choloroacetyl-epsilon-carbobenzoxy derivatives. Resolution was achieved enzymatically with cobalt-activated acylase I.

Drs. N. Izumiya (Visiting Scientist) and Winitz have prepared the four steroisomers of valyllysine anhydride and of phenylalanyllysine anhydride to obtain additional information on the comparison of chemical structure and biological activity in a group of compounds represented by the polypeptide antibiotics, gramicidin S and tyrocidin A. These antibiotics have the same features in common: a) a basic character due to the presence of one or more diamino acid residues; b) a cyclic conformation; and c) at least one amino acid residue of the D-configuration. Although the synthesized compounds also possess these characteristics, no antibiotic activity was found against either Gram-positive or Gram-negative bacteria. Moreover, they were not susceptible to the hydrolytic action of trypsin or chymotrypsin.

Nucleic Acids:

Drs. Sober and G. Rushizky (Visiting Scientist) are exploring modifications of chromatographic techniques that will permit separation of fractions containing large nucleotide sequences resulting from enzymatic digestion of nucleic acids. All of the mono-, di-, and tri-nucleotides resulting from ribonuclease digests of ribonucleic acids have previously been separated. Fractionation studies in collaboration with Drs. L. Heppel and M. Singer (NIAMD) of adenylic acid polymers from the hexa- to the

deca-nucleotide and larger fragments, coupled with analytical paper chromatography, have indicated the presence of contaminating enzyme activities in the original enzyme preparation used to degrade the polyadenylic acid polymer. Homologous oligonucleotides are being used as model substances for nucleotide separations. Such studies are of considerable importance in the investigation of nucleic acids in that they serve to characterize the enzymatic action, to characterize the nucleic acid itself in terms of its nucleotide distribution and sequence, and to obtain a variety of pure oligonucleotides which can subsequently be used in physical-chemical and enzymatic studies.

Drs. Rushizky and Sober have prepared pancreatic ribonuclease digest of a high molecular weight ribonucleic acid from yeast and from tobacco mosiac virus (supplied by Dr. C. A. Knight, Virus Laboratory University of California, Berkeley). The digests were fractionated by a two dimensional mapping procedure which combines paper electrophoresis and paper chromatography, and the fractions so isolated were further examined by physical-chemical and enzymatic methods. Comparisons were made with the complete digests. The following generalizations were obtained: a) oligonucleotides are released before the pyrimidine mononucleotides; b) with the pyrimidine mononucleotides, cytidylic acid was released before uridylic acid; and c) in each case, the cyclic intermediate of cytidylic acid or its oligonucleotides were converted to the terminal 3'-form before the corresponding uridylic acid compounds. Pancreatic ribonuclease has been found to have a preference for the enzymatic hydrolysis of pyrimidine linkages adjacent to purine linkages, i.e., these linkages are split before pyrimidine-pyrimidine bonds. This preferential specificity may be of considerable assistance in providing large and homogenous oligonucleotides in pure form which will aid in the determination of nucleotide sequences in nucleic acids.

As a further aid to work aimed at determining the structure of nucleic acids, Drs. Rushizky and Sober have purified two nucleases, "adenylase" and "guanylase," by means of conventional protein fractionation techniques, including precipitation and ion exchange chromatography. Both enzymes have been purified some 400-fold, and each is free of the other activity when tested at an enzyme-substrate ratio of 1:100. Since these two enzymes appear to hydrolyze adenylic and guanylic acid nucleotide linkages respectively, they will, especially when purified further, be particularly useful as tools for the specific cleavage of nucleotide significances of ribonucleic acid and provide therefore a complementary technique to that provided by the pyrimidine specificity of pancreatic ribonuclease.

Dr. Mary Maver, with Miss Antoinette Greco, has been purifying nuclease preparations derived from normal and neoplastic tissue, and has

further characterized their activities. Utilizing beef spleen and liver, rat liver, two transplantable rat hepatomas and a rat lymphosarcoma, she has obtained ribonuclease preparations by means of chromatography whose activities are characterized by: a) their pH optima on highly polymerized rat liver RNA; b) the products isolated from their hydrolysates when cyclic purine and pyrimidine nucleotides are used as substrates; c) the products of hydrolysis isolated in work with Dr. Rushizky from hydrolysates of yeast RNA by paper electrophoresis followed by paper chromatography; d) the products of their hydrolyses of polyadenylic and polycytidylic acids, and e) the effects of Mg ions and heat upon their RNAase activities. Two enzymatic activities which fractionate closely with the RNAase activities have been removed by repeated fractionation on carboxymethyl cellulose columns: a) an acid phosphatase which preferentially dephosphorylates the 3'-adenylic acid and, b) a phosphodiesterase, without RNAase activity, which hydrolyzes cyclic adenylic acid to yield the 2'-adenylic acid. These enzymes are much more active in calf spleen and liver and rat hepatoma 3683 than in normal rat liver.

An active "acid" RNAase with a pH optimum of 5.7 has been isolated which does not hydrolyze cyclic adenylic acid, but does hydrolyze cyclic cytidylic acid and polyadenylic and polycytidylic acids to yield the 3'-derivatives. With yeast or rat liver RNA as a substrate, both purine and pyrimidine linkages are broken and no core remains. The alkaline RNAase of spleen hydrolyzes cyclic adenylic and cytidylic acids to give the 3'-acids. Rat liver has been found to have a RNAase with a pH optimum of 6.5-6.7 besides the activities with optima of pH 5.7 and 7.3-7.8. The 6.5-6.7 RNAase is masked in crude preparations by an inhibitor which can be removed by treatment of the preparations with heat or para-chloromercuribenzoate. The RNAases of liver are considerably less active than those of other tissues studied.

Dr. Maver and Miss Greco have also been studying the sulfhydryl dependency of the RNAase activities of liver. As has been shown by others, para-chloromercuribenzoate (CMB) activates the alkaline RNAases by combining with an inhibitor. When this inhibitor has been removed by the chemical purification procedures used to obtain the more active nuclease preparations, often the alkaline as well as the acid RNAase activities can be activated (about 20 percent) by cysteine and inhibited by CMB. The acid RNAase is much more sensitive to CMB than the alkaline RNAases. The inhibitions of RNAase activities by CMB can be reversed by excess cysteine when the right quantitative conditions prevail. Spectrophotometric assays done according to Boyer show that the combination of the SH of the RNAase protein with the CMB is directly correlated in timed reactions with the inactivation of the enzyme. These studies also should not only aid in elucidating the nucleotide sequence of nucleic acids, but the sulfhydryl

sensitivity noted above may have important implications in the regulation of RNAase activity which may control RNA production for protein synthesis and in the liberation of energy-yielding or accepting nucleotides.

Extensive and detailed studies on deoxyribonucleic acid (DNA) and neutral deoxyribonuclease (DNAase I) have been made by Dr. J. Shack. Various conditions that affect enzyme activity (pH, substrate concentration, type and concentration of cations and of nonspecific salts) are not independent but rather are interdependent variables. Contrary to many earlier reports in the literature, it was found that proper control of these variables yields a substrate concentration-activity relation which corresponds to classical Michaelis-Menten kinetics. It was also found that the rate of hydrolysis of DNA falls off rapidly to a very low value when less than half of the susceptible bonds are broken. It appears that DNA contains linkages of widely different subsceptibility to the enzyme and that the rate falls off as the more susceptible linkages are broken, rather than because of reduction of substrate concentration or inhibition by products of the reaction. Comparison of results with native and denatured DNA indicates that differences in susceptibility are not dependent on the specific secondary structure possessed by native but not by denatured DNA, but are more probably related to the sequence of nucleotides in the vicinity of the particular bond.

Dr. R. Kielley, utilizing the assay method of Dr. Kornberg and associates, has characterized some of the conditions influencing deoxynucleotide kinase activities in liver homogenates of mice and rats and has studied the extent to which these enzymes can catalyze the synthesis of deoxynucleoside di- and tri- phosphates (apparently the direct precursor substances of DNA) in normal mature cells. From studies of substrate concentration on other deoxynucleotide kinase activities, one can conclude that the enzyme activity levels of deoxycytidylic acid, deoxyguanylic acid, and deoxyadenylic acid kinases are comparatively high and would not therefore be limiting factors in DNA synthesis. The activity of thymidylic acid kinase is somewhat low and additional work is required to determine if it can be considered a limiting factor in DNA synthesis. Preliminary results with tumor tissues indicate that the course of deoxynucleotide triphosphate synthesis in tumors is radically different in some respects from that found in normal liver.

Deoxycytidine diphosphocholine (d-CDP-C) and deoxycytidine diphosphoethanolamine (d-CDP-E) were isolated from the Novikoff hepatoma by Dr. W. C. Schneider two years ago. Assay conditions developed and isotopically labeled substrates synthesized have permitted initiation of studies of the factors responsible for the concentrations of these compounds which are higher in hepatoma and regenerating liver than in normal liver. The enzymatic activities related to the synthesis of d-CDP-C are considerably higher in homogenates of Novikoff hepatoma and of regenerating liver

than in those of normal liver. These compounds may prove to be of importance in DNA synthesis since they may lie on the pathway for conversion of ribonucleotides to deoxyribonucleotides.

In cytochemical studies of ribonucleoproteins of cells, Drs. R. Love, R. G. Suskind and N. P. Salzman have been able to distinguish nine types of ribonucleoproteins on the basis of structure, staining properties and functional behavior. A technique for staining the nucleolus metachromatically while other nuclear structures stain green has also been developed. Preliminary results suggest that when DNA synthesis in inhibited by 5-fluorodeoxyuridine riboside (FUDR), cell division is prevented, nuclear RNA synthesis is inhibited and cytoplasmic RNA synthesis is unaffected. After exposure to FUDR for 24 hours, RNA associated with the chromatin decreases, the nucleolus and nucleolinus are increased in size, and cytoplasmic RNA is increased.

With Drs. R. T. Bradley (last year an Anna Fuller Fund Fellow) and R. Roosa (now at the Wistar Institute), Dr. L. Law has provocative preliminary evidence obtained in three separate instances indicating that stable, DNA-induced transformations in mammalian cells may be demonstrable. P-388 sensitive cells incubated in vitro with DNA prepared from cells with a high level of 8-azaguanine resistance developed resistant colonies at a frequency 20 to 40 times that observed in sensitive control cultures.

Enzymatic Studies:

Dr. S. M. Birnbaum and Mr. M. C. Otey have made comparisons of the rate of disappearance of various D-amino acids in rats (interpreted as in vivo oxidation)and the rate of oxidation in vitro of corresponding D-amino acids. Small amounts of the compounds were force fed to young rats in 6 divided doses over a 2-day period. The excreta were quantitatively collected and were assayed for the D-amino acid. The total excretion subtracted from the amount fed was assumed to give a measure of the in vivo oxidation. Although the fraction "oxidized" varied from 40 to almost 100 percent, depending on the nature of the amino acid administered, this percentage value was nearly constant for a given D-amino acid at whatever level it was fed. Study of the specific and total oxidative activity of rat kidney and liver homogenates against a series of D-amino acids revealed: a) the relative specificity of the two tissues is similar if not identical in this respect, and b) there is no apparent relation between the in vitro and in vivo oxidative rates for D-amino acids. This latter observation raises some question as to the physiological interpretation of the significance of in vitro enzyme measurements.

In previous studies on the isolation of cell particulates by Dr. R. E. Greenfield, Jr., it was noted that the addition of the high molecular weight

polymer, polyvinylpyrrolidone (PVP), to solutions of sucrose resulted in a marked protection of cellular particulates. He has now made use of PVP-sucrose solutions and an interesting use of proteolytic enzymes administered intravenously to obtain suspensions of individual cells from different tissues. A suspension of single cells was obtained which contained 15 - 25 percent of the total liver nitrogen when the in vivo injection of trypsin and a fractional centrifugation procedure were used. This fraction was relatively free of cell debris and was made up predominately of parenchymal cells. The staining properties of the cells would indicate that a majority of them are alive: i.e., over 90 percent of the cells in most suspensions did not take up trypan blue or nigrosin and nearly all of the cells took up tetrazolium under anerobic conditions. The ease with which single cell fractions could be isolated from other organs varied markedly. Cells could be isolated in high yield from spleen, thymus, lymphosarcoma, and hepatoma. Only after multiple trypsin injections could the kidney be separated into cells, and even then the yield of cells was smaller than that found for the preceding organs. Following a single injection of trypsin the kidney separated into glomeruli and various lengths of tubules and blood vessels. Although muscle fibers which had been subjected to the in vivo trypsin injection could be easily teased apart, they had to be cut from the tendons. The brain and fibrous tumors separated into cells rather poorly, and the heart tissue did not separate at all.

Little change other than petechiae in the lungs could be seen on microscope examination of the organs following a single in vivo injection of trypsin. The animals did not appear in pain and remained healthy if permitted to survive.

When liver cell suspensions were kept at 0° in 20 percent PVP-10 percent sucrose solutions, the activity of aldolase, lactic acid dehydrogenase and catalase over an 8-hour period was nearly the same on a nitrogen basis as that found in the liver of origin. When the isolated liver cells were suspended in culture media at 38°, certain of these enzymes were lost. When 20 percent PVP-10 percent sucrose was added to NCTC 109, a protein-free, chemically-defined medium, 80 percent of the lactic acid dehydrogenase was lost from the cells to the media in one hour while over 90 percent of the aldolase and the catalase could be recovered from the cells. Aldolase was rapidly lost when sucrose was removed from the media. The addition of methocel or serum did not prevent the rapid loss of enzymes.

Drs. M. Chirigos and A. Goldin have studied a transaminase system, which mediates the transfer of alpha-amino groups of several alpha-amino acids to para-hydroxyphenylpyruvate with the formation of tyrosine, in liver and in spontaneous and transplanted tumors. With the amino acids employed, a low degree of similarity in transaminating pattern was observed

among the tumors when compared with the pattern for liver. Tumor extracts showed greater transaminating activity with glutamic acid than with glutamine, in contrast to liver preparations which exhibited similar activities with both compounds. The study also indicates enzyme systems not previously reported exist both in liver and in the tumors.

Dr. Helen Dyer has been studying glutamic-oxalacetic transaminase (GOT) activities of several tumors, including the Morris 5123 hepatoma, in a number of strains of rats and the activity in their sera. The level of serum GOT activity was not reduced during the first two months of feeding of several hepatocarcinogenic aromatic amines nor the chemically related nonhepatocarcinogenic compounds in the dosage used for the induction of tumors. GOT activity of livers of animals bearing tumor transplants did not differ significantly from those of normal animals. Although several transplanted tumors showed a lower GOT activity than normal liver, the Morris hepatoma 5123 had a higher activity. Furthermore, the GOT activity of sera of rats bearing hepatoma 5123 was considerably above that of normal rats, whereas the sera of rats bearing several other transplanted tumors was within the normal range. As the hepatoma 5123 grew in size, the serum transaminase activity increased accordingly. The serum level can also be raised by the intraperitoneal injection of the supernatent layer of a centrifuged homogenate of hepatoma 5123. Very little GOT activity was detected in the urine. The results suggest that the increased serum GOT activity originates in the tumor tissue and that it accumulates in the blood because it can be excreted only slowly by the kidneys.

An intriguing experiment has been performed by Dr. D. Burk and Mr. J. Hunter in which thermophilic chlorella cells were introduced into the peritoneal cavity of mice. Severe and chronic hypoglycemia occurred that resulted in the death of the animal. Cells washed from the peritoneal cavity of the mouse, where they had remained for 17 hours, grew vigorously when placed in proper medium, atmosphere and light. The hypoglycemia could be corrected by administration of glucose. This experiment followed studies on the inhibition of anaerobic and aerobic glycolysis of Ehrlich Krebs-2 mouse ascites cells by the presence of thermophilic chlorella cells grown simultaneously with the tumor cells in Krebs-Ringer medium. Because the algae consumed glucose at a rate several times faster than the cancer cells, the inhibition appears to result from low availability of glucose for the cancer cells. The algae, like the tumor cells, can produce lactic acid from glucose, but D-lactic acid instead of L-lactic acid is formed.

Dr. H. Kahler, with Mrs. B. Moore, extended his earlier studies on the pH of tumor tissue. Following administration of glucose to rats, the

pH fell rapidly and returned to normal in tumor tissue at sites in the tumor with good blood circulation. An estimate of the blood circulation was made by parenteral administration of Lissamine green dye; tumor tissue stains green if the tumor has a good blood supply, remains white if the blood circulation is poor, and remains red if there are pockets of blood in a static condition.

Drs. R. E. Madden and D. Burk have developed a procedure for comparing the metabolic behavior of tumors in the solid and ascites forms that may facilitate metabolic and chemotherapeutic studies of solid human tumors and may aid in establishing single-cell-cloned tissue cultures of a variety of normal and malignant cells from human and animal tumors. Single-cell preparations from several solid human and mouse tumors were prepared by stirring scissored tumor sections in media containing trypsin and deoxyribonuclease, centrifuging the strained supernatant fraction and taking the sedimented cells up in medium not containing the enzymes. With the animal tumors, comparisons were made with single-cell preparations from solid tumors and from the same tumor grown in the ascites form in terms of metabolic activity and viability (quantitative in vivo mouse inoculation studies). The behaviors of the two forms were essentially the same.

CHEMOTHERAPY INVESTIGATIONS

The search for chemical agents useful in the management of clinical cancer is under way on an unprecedented scale, and the stakes are high enough to warrant the tremendous efforts and large amounts of resources being expended. Two main approaches, not unrelated, have been employed, and, despite arguments to the contrary by proponents of one approach or the other, both need to be vigorously advanced. One, for the most part an empirical approach, involves screening of materials for their antitumor effects, the ultimate criterion being usefulness in the clinic. As yet, no satisfactory animal system screen has been developed that will predict the usefulness of drugs in the clinic, although some correlations exist between the responses of acute leukemia in children and leukemia L1210 in the mouse. Until much more data are available from studies on antitumor effects of various agents in both man and animal systems, it will be necessary to continue quantitative screening studies and to seek additional correlations. Investigation of many parameters must be continued, such as different agents of various classes, different tumor types (with more emphasis on nontransplanted tumors in animals), dosage schedules, routes of administration, combination therapy, immunological effects, and modifications of the host. The second approach, perhaps one in which the results might be of more far-reaching significance, but also perhaps slower in coming, is one aimed at understanding basic mechanisms of tumors, host-tumor relationships, and the mechanisms of action of chemical agents, both intrinsic and extrinsic, on the tumor and the host. Obviously, knowledge gained in one approach will often be helpful to the other, but until a satisfactory screen is attained, the second approach needs heavy support, perhaps comparatively more than it has received in the large-scale chemotherapy programs currently under way.

The Biochemical Pharmacology Section, under the leadership of Dr. A. Goldin, conducts extensive programs in both approaches of cancer chemotherapy, in the former through effective use of the contract mechanism for a drug development and evaluation program. In this latter, which is under the direction of Dr. C. G. Zubrod, Clinical Director, NCI, and under the supervision of Dr. Goldin and Messrs. J. M. Venditti and G. A. Brandner, particular attention has been given to two factors that have limited the success of cancer chemotherapy: (a) the toxicity of drugs for the host; and (b) the capacity of the tumor to develop resistance to therapy.

Relative to the former problem, the drug development and evaluation program is devoted primarily to determining quantitatively the relative antitumor specificity of drugs of potential clinical interest. In particular,

the program emphasizes structure-activity relationships among compounds which emerge as "active" from primary screens. Prolongation of the life-time of tumor-bearing animals is employed as the chief criterion for determining the relative therapeutic efficacy of drugs. Tumor size and body weight changes are, nevertheless, measured routinely. The program also investigates "active" compounds with respect to the optimal conditions for antitumor activity including the optimal schedule of treatment, route of administration, effect of combination therapy, etc. Such pharmacologic studies are needed to provide an accurate appraisal of the potential usefulness of a compound of interest.

Studies of the problem of the development of resistance to treatment have been facilitated by the emergence of active compounds of various chemical classes which have the capacity to inhibit growth at different biochemical sites and by the development of drug resistant sublines of tumors. The inclusion of studies of the effects of new compounds on drug resistant tumors is valuable from two standpoints. Such studies can uncover active compounds which may have clinical usefulness in cases which have become refractory to conventional treatment. In addition, drug resistant tumors are being utilized to explore possible mechanisms of drug action.

Tumor Assay Systems

In the past, three tumor assay systems have been studied most extensively:

The Advanced Leukemia L1210 Assay System:

To date, 213 compounds (88 of which were tested in 1960) have now been evaluated for their ability to prolong the life of mice with systemic L1210. The most active compounds against this tumor have been the halogenated derivatives of amethopterin. For example, 3', 5'-dichloro-amethopterin is about four times as active as amethopterin. Extensive structure-activity relationship studies among folic acid derivatives have shown that appreciable antileukemic activity is limited to the 4-amino derivatives of this metabolite. However, substitutions in various other positions (e.g. alkyl substitutions in the 10-position or halogenation in the benzene ring) can profoundly alter the degree of antileukemic effectiveness among the 4-amino derivatives. Cytoxan, a cyclophosphoramide nitrogen mustard, displayed activity equal to that of amethopterin. Uracil mustard and a number of ethyleneimines showed moderate antileukemic activity, but were only about 50 percent as effective as Cytoxan. Of 35 purines tested against advanced L1210, none appeared to be more effective than 6-mercapto-purine, which was 50 percent as active as amethopterin. Among the pyrimidines, 5-fluorouracil and its riboside displayed about equal

effectiveness (about 40 percent of the amethopterin effect). 4-Amino-pyrazolo (3, 4-d) pyrimidine was about 25 percent as effective as amethopterin. None of the various derivatives of this compound showed greater activity. Good activity was seen among the methylglyoxal-bis-guanylhydrazones. Three such compounds tested displayed activities of about 65 percent. Glyoxal-bis-guanylhydrazone, itself, was relatively inactive. Among the antibiotics, E-73 acetate, Streptovitacin A, and Actinomycin D were the most active, being about 30 percent as effective as amethopterin.

Special studies with the advanced L1210 assay system have been directed toward investigations of factors which may alter the host-tumor-drug relationship, including the influence of the route of administration, schedule of treatment, effect of therapy with drug combinations, etc. It has been shown, for example, that the efficacy of therapy with 3', 5'-dichloroamethopterin or 3'-bromo-5'-chloroamethopterin is substantially reduced when the drug is given orally. Consequently, the superiority of the halogenated derivatives over amethopterin, seen on subcutaneous administration, disappears when the drugs are given via the oral route. On the other hand, oral administration did not seriously reduce the effectiveness of 6-mercaptopurine. The methylglyoxal-bis-guanylhydrazones were only slightly less effective orally than subcutaneously. Studies of the influence of the treatment schedule on antileukemic activity showed that Cytoxan was more effective when given weekly than when given daily, twice daily, every four days, or every 11 days. In general, the activities of the antibiotics tested were not appreciably influenced by the treatment schedule. The methylgloxal-bis-guanylhydrazones were more effective when given daily than when given every four days or as a single treatment.

Combination therapy with amethopterin and Cytoxan showed that, with appropriate use of the drugs, treatment with the combination was superior to treatment with either drug alone. Urethane, which was inactive in itself, did not potentiate the activity of amethopterin, nitrogen mustard, or Cytoxan. 6-Azauracil, also inactive when employed alone, did not potentiate the activity of 6-mercaptopurine.

Increasing emphasis has been placed on studies of the effectiveness of chemical compounds on drug resistance. To date, these have included antipurine-as well as antifolic-resistant variants of L1210. Drug-resistant tumors are being used to uncover active compounds which may have clinical usefulness in cases which have become refractory to conventional treatment and to explore possible mechanisms of drug action.

The Sarcoma 37 Assay System:

A total of 70 compounds (26 tested in 1960) has been evaluated against early sarcoma 37 for ability to increase the life span of the mice. This tumor has been particularly insensitive to treatment and has therefore not been used routinely in the advanced form. Of the compounds tested against early S-37, alanine nitrogen mustard, Cytoxan, N-methylformamide and p-N, N-bis-(2-iodoethyl) aminobenzylphosphonic acid ethyl ester have provided appreciable increases in survival time. This tumor is of particular interest, because of the apparent lack of correlation between the degree of tumor inhibition and the increase in survival time attained as a result of therapy. For example, 6-mercaptopurine, Bayer 3231, and Actinomycin D were able to inhibit local tumor growth in mice with sarcoma 37 but provided little, if any, increase in survival time.

Special studies employing sarcoma 37 have been directed mainly to investigations of the influence of the treatment schedule on the effectiveness of compounds and to the influence of caloric restriction on local tumor growth and survival time.

The Adenocarcinoma 755 Assay System:

Unlike sarcoma 37, Adenocarcinoma 755 (Ca-755) has been quite sensitive to certain compounds, particularly the purine antagonists. In earlier studies in this program, 45 compounds were tested against early Ca-755. Extensive increases in survival time as well as tumor-free survivors were observed as a result of therapy with 6-mercaptopurine and some of its derivatives. The high degree of sensitivity of this tumor to therapy indicated that it could be employed more advantageously in an advanced form for the quantitative evaluation of drugs. Within the past year, 40 compounds have been tested for their ability to increase the survival time of mice with advanced Ca-755. Many purine derivatives elicited substantial increases, but none appeared to be more effective than 6-mercaptopurine. Among nonpurines, Cytoxan was the only compound which showed activity in the same range as 6-mercaptopurine. Moderate activity was observed for Actinomycin D and 4-aminopyrazolo (3, 4-d) pyrimidine.

Like sarcoma 37, Ca-755 displayed no correlation between local tumor inhibition and survival time extension. For example, three pyrazolo (3, 4-d) pyrimidines tested produced approximately 50 percent inhibition of the local tumor of early Ca-755 without providing substantial increases in survival time.

Other tumor systems that might be suitable for quantitative assay are under investigation.

Ehrlich Ascites Tumor:

Of eight compounds tested against the Ehrlich ascites tumor, only N-methylformamide provided an appreciable increase in survival time.

Hepatoma 129:

Cytoxan and 6-mercaptopurine were effective in retarding tumor growth and increasing survival time of mice inoculated subcutaneously with hepatoma 129 (obtained from Dr. Belkin), even when the treatment was withheld until the tumors reached about 1 cm. in size.

The Moloney Virus Leukemia:

The use of the Moloney virus for the production of indicator tumors in studies of the chemotherapy of leukemia in mice which is also being explored, has two advantages: (1) the induced neoplasm is a host tumor, and (2) the induced disease is similar to that which occurs spontaneously in nature. Drs. Goldin, J. Glynn and M. Chirigos and Messrs. J. M. Venditti and S. R. Humphreys, in collaboration with Dr. J. B. Moloney, have evaluated the therapeutic effectiveness of several drugs against this virus-host system, both with virus- and whole cell-induced leukemia. Drugs shown to be significantly effective in extending the latent period (time to death with leukemia) following inoculation of neoplastic cells are: Cytoxan, triethylenemelamine, and phenylalanine mustard (Sarcolysin); drugs of intermediate or no effectiveness are: Amethopterin, dichloro-amethopterin, 6-mercaptopurine, meticortilone and 5-fluorouracil. With virus-induced indicator tumors, the following drugs were moderately effective: Cytoxan, methotrexate, and methylglyoxal-bis-guanylhydrazone. The last-named compound produced marked reduction in spleen and thymus gland weights.

Spontaneous Tumors:

Because of the poor correlation between results in animal transplantable tumor screens and in man and because of the growing body of information that indicates transplantable tumors are usually very different from spontaneous ones, screens involving spontaneous tumors need to be established in spite of the increase in variability among the experimental animals and the longer holding time and greater numbers of animals required. Drs. Goldin and Chirigos and Messrs. Venditti, Humphreys and N. Mantel have initiated studies testing the effectiveness of 3', 5'-dichloroamethopterin, 6-mercaptopurine and cytoxan on a spontaneous mammary tumor in strain C3H mice. As might be expected, the untreated control animals showed considerable variability from day of entry into the

experiment to the day of death (median, 40 days; range, 5 to 117 days). Survival time was not increased although some retardation of tumor growth was observed. The median survival times observed suggested that drug toxicity was limiting with cytoxan and 6-mercaptopurine in the doses employed.

In spite of logistical difficulties, some data on therapeutic effectiveness of several agents against spontaneous tumors in noninbred experimental animals perhaps should be obtained as another comparative screen of importance to clinical cancer.

Biochemical Studies:

3'-Fluoroamethopterin and 3', 5'-difluoroamethopterin were tested against leukemia L1210 by Drs. A. Schrecker, J. Mead and Goldin and Mr. Venditti. The former is comparable in effect to methotrexate and the latter to the dichloroanalog. These findings suggest that the greater effectiveness of 3', 5'-dichloroamethopterin over methotrexate in L1210 is not due to steric hindrance of the halogen atoms, but to the electronegative nature of the halogens. 3', 5'-dimethylamethopterin will be synthesized to test further the concept.

A simple fluorometric method has been developed by Drs. Chirigos and Goldin for the determination of small amounts of phenylalanine mustard (Sarcolysin), and time studies on the distribution of the drug following its administration to tumor-bearing animals have been done. Binding of the drug by cellular fractions of the blood was indicated.

In collaboration with Dr. N. O. Kaplan (Brandeis University), Dr. Goldin and Mr. Humphreys have studied several precursors, analogs and antagonists of diphosphopyridine nucleotide (DPN) and related compounds for their influence on DPN metabolism, their ability to reverse antileukemic activity of certain drugs and their antitumor effects. Dose response curves have been obtained on some 50 compounds, with structural differences producing a wide range of toxicity. Only 6-aminonicotinamide, beta-acetylpyridine and the thiadiazoles have evidenced any antileukemic effects. Nicotinamide has been shown to reverse the antileukemic action of 2-amino-1, 3, 4-thiadiazole and 2-ethylamino-1, 3, 4-thiadiazole. As a follow-up on the earlier finding that nicotinamide administration in the mouse led to more than a ten-fold increase in liver DPN, other compounds have been shown also to result in rapid formation of liver DPN: 3-acetypyridine, pyridyl-3-methyl carbinol, beta-picoline, monomethyl and monoethyl nicotinamide.

As an example of work representing a continuation of studies reported last year on the mechanism of action of antifolic drugs, investigations are summarized on biochemical studies of resistance to antifolics in two resistant cell lines (C82 and M66) derived from leukemia L1210. Drs. Schrecker, Mead and Goldin and Mr. Humphreys, in collaboration with Dr. M. Friedkin (Tufts University), have found that both the local tumor and the spleen of the M66 variant had the same amount of dihydrofolic reductase activity as the sensitive L1210 leukemia, while in the C82 subline the level of enzyme activity was increased thirty-fold. Formate-C^{14} incorporation into the acid-soluble adenine of spleen and local tumor was inhibited by antifolics to a much lesser extent in the C82 variant than in the sensitive L1210 line. With single treatment with methotrexate or 3', 5'-dichloroamethopterin, some inhibition was still obtained in the C82 line. With daily treatment, however, no inhibition at all was observed after several days of treatment. This would suggest that there are still some sensitive cells present in the C82 antifolic-resistant line and that they are eliminated early in treatment. It tends to support the hypothesis that, in the C82 subline, there is a mixed population of cells with varying amounts of dihydrofolic reductase, the larger proportion of cells having higher amounts of the enzyme. The cells that do not possess high activity may be the ones eliminated during the first few days of treatment. Whether in the original L1210 line some cells with high dihydrofolic reductase activity are already present has not yet been determined. In the M66 line, inhibition of formate incorporation by methotrexate or 3', 5'-dichloroamethopterin (single treatment) is of the same order as in the sensitive line. At present the effect of multiple treatment in the M66 line is under study. Preliminary results indicate that, here again, treatment induces drug tolerance. It would appear, so far, that the mechanism of resistance may be different in the M66 variant from that in the C82 variant. In the latter a high enzyme activity produces a high tolerance for the inhibitor.

Drug Resistance Studies:

Dr. Law continues his studies on the mechanisms involved in inhibition of leukemic cell growth by drugs and in development of drug resistance. A subline of the lymphocytic neoplasm L1210 has been developed which is resistant to the effects of 6-mercaptopurine, 5-fluorouracil and amethopterin. The growth rate and strain specificity have become altered. Comparative studies with the parental cell line on the effects of several new agents are in progress.

With Dr. M. Lane (now at Baylor University), Dr. Law has found that the development of resistance to 5-fluoropyrimidines apparently has changed the sensitivity to cyclophosphoramide (Cytoxan); "resistant" lines (to three other compounds) were found to be more sensitive to cyclophosphoramide than the "sensitive" parental line. At optimal dosage levels,

complete regressions of established tumors in both P-815 and L1210 lines have been observed. In collaboration with Dr. E. P. Anderson and Dr. W. Brockman (Southern Research Institute), strains of both these cell lines that are resistant to 5-fluorouracil were found to have decreased capacity (or decreased rate) of nucleotide formation. In contrast to findings reported by Reichart, phosphorylase and kinase activities, which probably constitute the major pathway for the biosynthesis of uridylic acid in mammalian tissues, are found in these resistant lines. Preliminary results indicate both enzyme activities are weaker in the resistant lines compared with the sensitive lines.

Previous studies with intact cells and cell-free extracts by Dr. Anderson on the biochemical effects of azaserine in azaserine-sensitive and azaserine-resistant cell lines of the plasma cell neoplasm 70429 indicated an "intact cell factor" was operative in the resistance mechanism, but to learn if this "factor" was related to greater impermeability of the resistant cells to the drug, it was necessary to determine specifically the entry of the compound into the cells. In collaboration with Dr. J. Jacquez (Sloan-Kettering Institute), Dr. Anderson has studied the capacities of the two cell lines for concentrative cell transport and intracellular breakdown of azaserine. No major differences were found in the capacity to concentrate azaserine within the cell. Quantitative differences, however, were found; the sensitive line showed a slightly higher initial velocity of uptake, whereas, over a longer period, the resistant cells surpassed the sensitive ones in the final intracellular concentration which they could maintain. No appreciable differences were found in the extent of intracellular breakdown of the azaserine. Additional studies have been started on reversal of azaserine transport by normal amino acids in the two lines, since pool size of normal metabolites and their capacity to reverse transport of the antimetabolites are also important considerations in understanding the mechanism of development of resistance. Some difference in pool size of nucleotides have also been indicated by data already collected.

Glycolysis Studies:

In previous Annual Reports, Drs. M. Woods and D. Burk reported that a high degree of malignancy is associated with greatly increased glycolytic capacity and lowered sensitivity to glycolytic inhibition of the anti-insulin type. In general, those experimental neoplasms in which the hexokinase reaction is under strong insulin-anti-insulin control respond to one or more chemotherapeutic agents better than do those cancers in which glycolysis is less readily inhibited by compounds of the anti-insulin type.

Human normal and leukemic leucocytes have been studied with special reference to their glycolytic responses to anti-insulin steroids, insulin and endotoxic polysaccharides by Drs. Woods and Burk and Mr. J. Hunter. Results obtained to date, though not conclusive, indicate that glycolysis in lymphocytic cells (normal and leukemic) is much more sensitive to inhibition by steroids than is the case with cells of the myelocytic series. The data indicate that insulin: anti-insulin regulation of glucose metabolism plays an important role in the metabolism of leukocytes (lymphocytic and myelocytic), and that endotoxic polysaccharides counteract such glycolytic inhibition in a manner resembling, although not identical with, insulin.

During the past year, studies on the action of fluorinated pyrimidines and related compounds on tumor glycolytic metabolism were extended. In collaboration with Dr. J. Seitz (Visiting Scientist from the Institute for Hematology and Blood Transfusion, Leningrad), the effects of fluorinated pyrimidines on "high energy phosphate" metabolism of tumor cells were studied. At low levels of inorganic phosphate, where in vitro glycolytic inhibition may reach 80 percent, no reduction in net acid-insoluble high energy phosphate was observed. All of this phosphate fraction, however, may not have consisted of simply ADP and ATP, but might have involved possible fluorinated derivatives.

While inhibitory action by a series of 5-fluorinated pyrimidines seems to depend on the presence of an active glucose metabolism during exposure to the drug, in a few instances, following in vivo exposure to 5-fluorouracil, the transplantability of ascites tumor cells was markedly inhibited without concomitant glycolytic inhibition. However, in all instances, there was a strong positive correlation between the in vitro glycolytic inhibitory activities of a series of substituted pyrimidines and their in vivo anti-tumor activities.

Evidence was obtained that the anti-glycolytic pyrimidines inhibit aerobic glycolysis in ascites tumor cells by competitively inhibiting the utilization of inorganic phosphate during initial stages of exposure to the drug. A secondary phase of inhibition is not reversed by increasing the concentration of inorganic phosphate in the medium. A recent report by Lemon is of interest in this connection: simultaneous intravenous administration of glucose with 5-fluorouracil in man is accompanied by reduction in host toxicity, with retention of anticancer effects.

Cytoxan, a cyclic phosphoramide derivative of nitrogen mustard, was found to inhibit tumor cell glycolysis. Comparison of the L1210 ascites strain resistant to cytoxan, developed by Dr. Lane with the L1210 ascites susceptible strain, showed a difference in the glycolytic and

respiratory properties of the two strains with respect to cytoxan action. Cytoxan inhibited the glucolysis and respiration of the susceptible strain considerably more than in the resistant strain, the glycolysis and respiration of the tumor being determined after action of cytoxan in vivo. Nicotinamide and diphosphopyridine nucleotide reversed cytoxan action in the resistant more than in the susceptible strain. Further progress on the activation of cytoxan in vitro was made. In the presence of triphosphopyridine nucleotide (TPN) and magnesium, as well as adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN), cytoxan inhibition of glycolysis of liver and mammary tumor homogenates and derived subcellular elements was obtained. Thus a connection of cytoxan action with magnesium and TPN was found. An inhibition of ATP-ase by 5-fluorouracil was demonstrated for the L1210 ascites tumor cells. Further study of the respiration and glycolysis of tumors and normal tissues was undertaken. Glucose usually produced a pronounced Crabtree effect (inhibition of respiration by glucose) in the case of ascites tumors, but this was not always true. TPN, with increased magnesium and nicotinamide, increased the glycolytic production of carbon dioxide three to four times above the amount produced in the presence of ATP and DPN only, in mouse mammary tumor homogenates, but not in liver homogenates. An increase in the amount of lactic acid produced was also found. This finding has been extended to whole cells for the thymoma ascites. The increased glycolysis in the presence of TPN is not so great with cells, but clearly demonstrable. There is no evidence to support the hypothesis that DPN is formed from TPN. Other nucleosides such as inosine, uridine, cytosine and guanosine triphosphates could be substituted for adenosine triphosphate partly or wholly in tumor glycolysis, in studies with thymoma ascites and mammary tumor homogenate.

Chemotherapy of Experimental Central Nervous System Leukemia:

The clinical studies of Drs. E. Freireich and L. Thomas in the General Medicine Branch have revealed the importance of central nervous system involvement as a serious complicating factor in chemotherapy of clinical leukemia. Similar findings have been seen in mice treated sufficiently to give extensive increases in survival time. An experimental animal model for the study of central nervous system leukemia has now been developed by Drs. Goldin, Chirigos and Thomas and Mr. Humphreys. Treatment with amethopterin or 3', 5'-dichloroamethopterin increased the survival times of the mice inoculated intracerebrally with L1210 even when the therapy was initiated as late as a few days prior to the death of the control animals. The effectiveness suggests that these drugs may be capable of crossing the blood-brain barrier in sufficient quantity to exert direct antileukemic action in the brain. Cytoxan was found to have limited effectiveness against intracranial leukemia.

SERVICE FUNCTIONS

The many research contributions made by the investigative staff would not be possible without the very considerable assistance given by many others on the staff of the Institute. Messrs. W. Magruder and J. Murphy continue to provide invaluable assistance in meeting those many difficult day-to-day problems which must be handled expeditiously if research progress is to continue at a good pace. The skills shown by them are not only of tremendous importance to the research programs, but in a very real sense, often require a creativity just as unique as that needed for research.

The administrative clerical and secretarial assistance provided throughout the Institute, likewise, is in a very real sense a contribution to the programs of the Institute without which much of the work could not come to fruition.

The Pathologic Anatomy Branch, under the leadership of Dr. Harold L. Stewart, continues to perform very superior work in pathologic anatomy, not only for studies in the NCI but for investigations throughout the NIH. Pathologic anatomy work-up of surgical specimens (2,521 specimens in 1960) and of autopsy material (276 autopsies during the year), including the extensive discussions held by the pathologists with other members of the staff, provide basic pathology information of the highest quality for the clinical studies under way at NIH. Nearly 50 scientists in the various laboratories at NIH have requested particular tissues from surgical and post-mortem services during the past year and, on many occasions, it was possible to furnish these investigators with fresh human material.

Six full-time residents in the Department of Pathologic Anatomy are receiving excellent advanced training in pathology.

The Cytodiagnostic Service, under the supervision of Drs. R. A. Malmgren and E. W. Chu, provides excellent exfoliative cytology service to NIH and constitutes a major element of the diagnostic facilities available to the clinical staff (10,161 slides, representing 3,055 accessions, were examined during 1960). The Service has participated in quantitative studies on surgical wound washings, on fluids draining from such wounds, on body fluids such as spinal fluid, and on peripheral blood, utilizing both the Papanicolaou staining procedure and special filtration techniques for the collection of cells.

Mr. J. Albrecht and his staff of histopathology technicians continued to provide the excellent service that the research staff has learned to

reply on from this superior group. They prepared 129,068 stained slides for the staff of NIH, of which 29,994 required special staining. The fine on-the-job training given to technicians, several of whom later work closely with members of the investigative staff, continues to pay dividends for the National Institutes of Health.

The analytical chemistry group in the Laboratory of Biochemistry under Mr. R. J. Koegel's supervision continues to provide high quality service. During 1960 the character of the requests changed significantly, with a reduction in requests for microchemical empirical elemental analyses, but with an equal workload of some 5,500 individual analyses.

The intramural programs of the National Cancer Institute constitute a unique resource in the battle against cancer. Great research institutions are not created nor maintained easily, and it behooves us to do all that we can to insure that those elements that have made the National Cancer Institute a leading research organization in the field of cancer are maintained, nurtured and cherished. This is not easy to do in the face of myriads of regulations and procedures often brought into being for reasons unrelated to research, but also often produced by ourselves as part of a Parkinson's Law expansion invoked to meet our problems. We should, rather, insist on the elimination of some of the self-generated problems. As we have become larger, many of these problems have accumulated, perhaps faster than at an arithmetic rate. The answer to these accumulating problems is not, I submit, addition of larger centralized staffs and the larger accompanying mechanisms that go with such additions, but rather a decentralization of decision-making and real authority appropriate for the respective administrative echelons.

Respectively submitted,

Carl G. Baker

Carl G. Baker, M. D.
Assistant Director (for Research)
National Cancer Institute

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Serial No. 201
Office of Associate Director
in Charge of Research
National Cancer Institute
Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies of Cytochemical Changes in Living Individual Cells

Principal Investigator: George Z. Williams, M.D.

Cooperating Units: Clinical Pathology Department, Clinical Center

Field Investigations and Demonstrations Branch
National Cancer Institute, Serial No. 144

Department of Chemistry, University of Richmond
Dr. J. Stanton Pierce, NCI Grant No. C-2693

Man Years
Total : 1.8
Professional: 0.5
Other : 1.3

Patient Days: 0

Project Description:

A. OBJECTIVES

The study of changes of chemical states and activities in living individual cells by cytochemical methods. Ultraviolet microscopy constitutes the chief instrument for analysis. The image is detected and transmitted by TV to a monitor for direct observation, and the intracellular reactions and structures and their ultraviolet absorption characteristics are depicted and measured on a line-selecting oscilloscope. During the past calendar year this work has included:

1. Further improvement of the ultraviolet television microscope and oscilloscope, synchronization of the components for time-lapse cinematography and a reference calibration signal device for control of accuracy and reproducibility.

2. Development of a precise, reliable, variable-time, high speed electronic shutter for the ultraviolet beam.

Part B included Yes

3. Observation and photography of the UV absorption characteristics of normal and abnormal living cells to further establish base lines for cytochemical techniques; these included human leukocytes, mouse liver cells, and several lines of tissue culture cells.

4. Completion of a systematic survey of properties and reactions of tetrazolium salts for use in study of intracellular reactions.

5. Time lapse UV cinematography of human leukocytes and their reaction to controlled short sequential exposures of UV light.

B. METHODS

Television Ultraviolet Microscopy

The application of ultraviolet television microscopy to the investigation of chemical reactions in living individual cells is accomplished by a minimum flash exposure of the cell to ultraviolet, storage of the ultraviolet image on the blanked (temporarily inoperative) photoconductive face of the television vidicon and subsequent and synchronous collection of this picture with the electron beam, amplification and display on the kinescope monitor. Time-lapse motion picture photography and oscillography record the density changes. This technic permits oscillographic measurements of ultraviolet absorption of structures and chemical reaction products in living cells. For reliable measurements of density, continuous control and calibration of the electronic components was necessary and the calibration signals introduced into the circuit last year were evaluated during the present year and found to be a highly satisfactory method for controlling operation of the equipment.

One fault plaguing the proper operation of this method was the failure of the mechanical shutters (available on the market) to reproduce exact opening times for the ultraviolet light. Therefore, an electronic shutter was designed and fabricated in the Instrument Branch and evaluated. This shutter employs two dynamic audio speakers. To the center of the voice coil diaphrams is attached a spindle carrying a rectangular shutter blade. These two blades oppose each other, overlap and are so controlled electronically that one opens and the other closes at a given time interval to produce the desired exposure. The mechanical delay of the voice coils is about 1-2 milliseconds and light pulses of from 5-75 milliseconds duration are available. For long periods of continuous operation, the open time at a given setting is constant within 5% or less. This is a very satisfactory shutter and will be described for publication in a technical journal.

There is some continuing variation of the brightness level of the kinescope picture due to the variation of the heating and cooling of the vidicon sensitizing lamp. This will be corrected by developing a constant current power supply for this small lamp.

Upon reducing the ultraviolet exposure time to 10 milliseconds, dark margins, upper and lower, were found on the kinescope picture, and analysis

proved that this was due to the vidicon blanking pulse which was not a true square wave. This has been corrected to yield a very nice flat uniformly illuminated kinescope picture field. It is now possible to obtain good pictures by motion picture time-lapse photography at ultraviolet flashes of monochromatic 265 mu ultraviolet light which represents an amount of light approximately 1/1000th of that necessary to take a photograph directly on film.

A new vidicon tube has been developed by the engineers, Mr. Veith and Mr. Neuhauser, at the RCA Tube Plant and one has been made available for evaluation in our laboratory. This tube utilizes a different photoconductive material than that used before and is more sensitive to short pulses of light and therefore, is more ideal for our purposes.

A further improvement in the resolution and clarity of pictures as well as increased transmission of ultraviolet light through the optical system has been achieved by purchase of a set of new Zeiss quartz-fluorite achromatic ultraviolet objectives and condenser. These have been tested and found superior to any reflecting type of quartz lens heretofore available. They are achromatic from about 250 mu to 700 mu with the proper thickness of quartz slide. Their transmission of ultraviolet in the far region is much superior to other lenses of both reflecting and transmission types, and this has improved the sensitivity of the instrument as well as the resolution of the resulting photographs.

Cell Assay System

Individual liver cells carefully separated from mouse or rat liver, tissue culture cells, and human leukocytes from peripheral blood, are being utilized as the cell assay systems.

All individual cell studies have been controlled with test tube experiments utilizing similar concentrations of tetrazolium in suspensions of several million cells per milliliter. To quantitate tetrazolium reduction in these volumes it is necessary to accurately enumerate the cells. This was accomplished by applying the method developed and described last year, namely counting and sizing the cells with the Coulter Electronic Counter. The work on evaluating this instrument and developing the method of counting and volume-sizing large numbers of cells in suspensions, was completed and the report published in the Journal of the National Cancer Institute. We use this method routinely to count all cell suspensions for tetrazolium experiments.

Tetrazolium Salts

Work on determining the properties of tetrazolium salts and their formazans has been completed. In addition to the ultraviolet and visible light absorption maxima and extinction coefficients of each one of the compounds tested, we also determined their reaction to the DPNH diaphorase system, the "spontaneous" or endogenous reduction of these compounds by living cells, (liver), and their reduction in the presence of the added

substrate, succinate. The findings are tabulated and briefly described under MAJOR FINDINGS. From the grouping of tetrazoliums in accordance with certain structural, chemical and biological reduction properties, we have been able to select 8 tetrazoliums representing the three groups of non-nitro, mono-nitro, and di-nitro salts which possess the optimum characteristics for use in biological studies.

C. MAJOR FINDINGS

Instrumentation

A precise high speed short interval electronic shutter has been designed, fabricated and evaluated. Although it is only in the "bread board" status, it works very nicely, reproducing exact 10 millisecond opening times, repeatedly at 5, 10, 15, 30 second cycles or at 2 or 3 minute cycles. It is continuously variable for opening times of 5-75 milliseconds. The resulting transmitted light wave is almost a square wave as seen on the oscilloscope, indicating that the opening and closing times are very short compared with the open periods.

The new Zeiss achromatic ultraviolet transmitting objectives and condenser are excellent. The photographs produced are clear, of high resolution and excellent enlargements can be made, producing magnifications in the order of 30,000 diameters from ultraviolet at 265 mu.

Tetrazolium Salts

Several additional tetrazolium salts were synthesized by Dr. Pierce (University of Richmond), to complete the series of nitro and methoxy substituted groups on the phenyl rings. The relative reactivity correlates with the presence or absence of nitro groups in the para, ortho, or meta positions of the phenyl rings. The attached table lists the physical and certain biological reduction properties in the order of phenyl ring substitutions of similar structure, non-nitro, mono-nitro, and di-nitro. The maximum ultraviolet and visible light absorption of the tetrazolium and formazan salts are reported as well as the molar extinction coefficients which vary from 4,000 to 18,800 and does not seem to be correlated with any particular structure.

Reactivity of Tetrazoliums

It will be noted that there is a distinct correlation of reduction properties with the presence or absence of the nitro groups, their number and position. The non-nitro compounds fail to reduce the DPNH diaphorase system, show no endogenous reduction in the liver cells, and are reduced by liver cells supplied with excess succinate at very low rates. There is one exception in this group, that of the chloro compound which reduces very well in the presence of succinate, but methoxy substitutions have no effect of increasing the reduction sensitivity. The mono-nitro compounds average 16×10^{-9} moles per minute of formazan in the DPNH diaphorase system in which

there is no reduction of non nitro compounds. The di-nitro compounds show much more reduction by the DPNH system, $56x10^{-9}$m per minute. There is none to a very slight (0.5) endogenous reduction of mono nitro tetrazoliums without substrate, but excellent reduction (0.2-1.0 u moles per $5x10^5$ cells) with added succinate. Somewhat more endogenous reduction (0.07-0.17) occurs with the di nitro salts and about the same amount of reduction as the mono-nitro group with added succinate, 0.3,-0.7um per $5x10^5$ cells.

The effect of temperature on reduction with succinate substrate by fresh liver cells is manifested by a marked decrease for seven typical tetrazolium compounds at temperatures of 23°C. The reduction at 37° and 45°C are almost identical for all compounds except Nos. 42 and 45. 42 is a mono-nitro orthomethoxy tetrazolium and 45 is the non-nitro chloro tetrazolium. The reduction of these two was markedly decreased at 45°C. The reason for this is not apparent.

Reduction of tetrazolium to formazan is related to cell concentration. The liver cells were counted and six tetrazoliums, two from each of the three groups were compared. The reduction at 5, 10, 15, 30, and 60 minutes was measured by spectrophotometry extraction of the formazan. In each instance the amount of formazan produced is proportional to the concentration of cells, except for No. 38, a di-nitro compound, with which reduction is so marked and rapid that almost as much formazan is produced by $1.5x10^5$ as by twice that number and only about 30% more formazan was produced by $6x10^5$ cells. For the other salts, the amount of formazan produced was directly proportional to the number of cells.

The rate of reduction of tetrazolium by liver cells also is related to the concentration of the tetrazolium salts in the succinate-buffer mixture. In all experiments it was found that the rate of reduction increases in a linear fashion with the exception of No. 38, and No. 40 which increase very rapidly up to 10 or 15 minutes and then little additional reduction occurs. This was the highest rate of reduction, the other tetrazoliums showed rates proportional to the concentration of the tetrazolium from 0.0012 to 0.005 molar. The rates of the non-nitro compounds were much less than those of the mono-nitro compounds. The di-nitro compounds showed some higher rate of reduction than the mono-nitro.

Several experiments were conducted to determine the nature and rate of cell permeability to 3 characteristic tetrazoliums, Nos. 38, 60, and 45 representing the three groups. For FY 60, we reported that results of permeability studies utilizing a chemical reducing substance after cells had been incubated in tetrazolium salts and washed revealed rapid and free permeability of the cell walls to tetrazolium. Subsequent experiments utilized the succinate-substrate system. Liver cells were incubated in optimal concentration of tetrazolium for 30 minutes at 38°C., centrifuged, washed once with buffer, and suspended in succinate-buffer. It was assumed from previous evidence that the cell wall is freely permeable to sodium succinate in concentrations of 0.034 moles. Control experiments were conducted to determine the amount of endogenous reduction without the presence of succinate. The difference between formazan produced by control and succinate

supplied cells was plotted for periods of from 5 to 30 minutes. Except for the non-nitro tetrazoliums, there is a reduction of residual tetrazolium which apparently remains in the cell after washing. The reduction curve from 0 to 30 minutes for cells in the presence of tetrazolium and succinate (control) is characteristically rapid and produces large amounts of formazan for the di-nitro compound; (i.e., 1 uM in 30 minutes), while cells incubated in tetrazolium 30 minutes, washed, and incubated later in succinate produced much less formazan; about 0.18 uM in 30 minutes or less than 20% of the control. Other experiments with di- and nitro- tetrazoliums produced similar results. Because direct observation under the ultraviolet of liver cells in succinate-tetrazolium-incubation mixtures reveals progressive reduction to formazan in the vicinity of mitochondria inside the cytoplasm without the appearance of formazan outside the cell or in the vicinity of the cell surface, it is concluded that the results of the present experiment indicate a rather free permeability to the cell wall to the tetrazolium. It is suggested that all but a certain amount (20%) bound to cellular protein is easily washed out with one washing. Further experiments will be necessary to elucidate this observation.

Studies with Human Leukocytes

Several time-lapse motion pictures have been completed recently to determine the resistance or sensitivity of human leukocytes to the short interval exposures to ultraviolet. Preliminary results indicate that with the ten millisecond exposure at intervals of 15 seconds, human leukocytes may tolerate ultraviolet exposure at 265 mu for periods of 15 to 30 minutes before showing structural and dynamic changes of damage, such as rounding of the cell and cessation of ameboid activity. Severe damage is manifested by sudden and marked decrease in the density of the nucleoproteins in the nuclear bodies, vaculation and swelling of the cytoplasm and sometimes sudden rupture of the cytoplasm with dissemination of its contents.

D. SIGNIFICANCE TO THE NATIONAL CANCER INSTITUTE

It has been shown that quantitative data of reaction rates of an enzyme system causing the reduction of tetrazolium to formazan can be measured over periods of time in living cells. This permits accumulation of information about dynamic changes in metabolic activities with a minimum change in the environment of the living cell system. This type of study utilizing ultraviolet television microscopy and time-lapse cinematography for determination of reaction rates in living intact cells will contribute to our knowledge of the chemical systems therein. We propose to apply these methods to the study of chemical systems in normal and neoplastic cells and their responses to agents such as chemical inhibitors and irradiation.

E. PROPOSED COURSE

1. Investigation will be continued to better determine the mechanism of reduction of several of the better tetrazolium salts by living cells and

to study the possible specific activities of each in relation to various substrates.

2. Further experiments will be conducted to determine the permeability of the cell walls to tetrazolium and the difference between such cells as tissue culture and liver cells.

3. With the constant reproducible ultraviolet light shutter activity and a controlled light source, it will now be possible to study in a more quantitative fashion, the patterns of tetrazolium reduction within living cells over a period of time by timelapse photographs and density changes indicated by oscillographs.

4. The nature and time of onset of injury to cells by ultraviolet including human leukocytes, tissue culture cells, and mammalion liver cells will be determined to establish a base line for evaluating effects of other agents such as tetrazoliums and leukocidin.

5. Tetrazolium reduction by human normal and leukemic leukocytes will be determined and compared.

6. A system of different substrates will be utilized and compared for tetrazolium specificity in cell chemistry.

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Office of Associate Director
in Charge of Research
National Cancer Institute
Bethesda, Maryland

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Individual Project Report
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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Williams, G. Z. and Peacock, A. C.: Ultraviolet Studies of Tetrazolium Reduction in Living Cells. Experimental Cell Research, Suppl. 7, 131, Nov. (1959).

Peacock, A. C., Williams, G. Z. and Mengoli, H. F.: Rapid Electronic Measurement of Cell Volume and Distribution. J. Nat. Cancer Inst. 25:63, July (1960).

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF CERTAIN TETRAZOLIUMS

Physical Properties										
Code No.	M. W.	Phenyl Groups			Melting Point °C	Light Absorption (Å)				Molar Extinction Coefficient (490 mu)
						Tetrazolium		Formazan		
		C_5	N_2	N_3		U. V.	Visible	U. V.	Visible	
None No_2										
4	336	–	–	–	243	2500	–	2950	4850	14,800
8	366	CH_3O	–	–	227-229	2715	–	2975	4950	9,900
5	380	OCH_2O	–	–	170-181	2250	–	3000	5000	10,800
17	326	C_5H_{11}	–	–	209-211	2650	–	2550	4120	6,650
6	366	–	–	OCH_3	214-215	2425	–	3000	4800	15,000
44	396	CH_3O	–	OCH_3		2750	–	3000	5000	10,490
54	410	OCH_2O	–	OCH_3		2220	–	3100	5100	10,000
45	372	–	–	Cl		2450	–	2950	4860	10,940
Mono No_2										
49	412	–	No_2	OCH_3		2450	–	3500	4800	10,000
42	412	–	No_2	O-OCH_3	204-206	2550	–	2620	4970	15,400

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF CERTAIN TETRAZOLIUMS

Physical Properties										
Code No.	M. W.	Phenyl Groups			Melting Point °C	Light Absorption (Å)				Molar Extinction Coefficient (490 mu)
						Tetrazolium		Formazan		
		C_5	N_2	N_3		U. V.	Visible	U. V.	Visible	
Mono No_2(cont)										
14	412	CH_3O	No_2	–		2750	–	2580	4880	15,200
7	425	OCH_2O	No_2	–	228-229	2260	–	3750	4900	12,200
21	441	CH_3O	No_2	OCH_3	221-222	2750	–	3600	4880	18,800
23	456	OCH_2O	No_2	OCH_3	184-198	2750	–	2600	4900	16,970
13	412	CH_3O	–	No_2		2750	–	2900	4880	14,800
60	442	CH_3O	–	p-No_2 O-OCH_3		2750	–	3000	5150	12,900
43	412	CH_3O	–	m-No_2		2700	–	2800	4800	10,000
39	412	–	–	O-No_2 p-OCH_3		2410	–	2250	5180	5,600
59	442	CH_3O	–	O-No_2 p-OCH_3		2740	–	3000	5400	4,000
58	380	No_2	–	–		2760	–	2550	4725	9,600
51	412	No_2	–	OCH_3		2800	–	2525	4720	15,125

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF CERTAIN TETRAZOLIUMS

Physical Properties										
Code No.	M. W.	Phenyl Groups			Melting Point °C	Light Absorption (Å)				Molar Extinction Coefficient (490 mu)
						Tetrazolium		Formazan		
		C_5	N_2	N_3		U. V.	Visible	U. V.	Visible	
Di-No_2										
50	426	–	No_2	No_2		2590	–	2500	4240	18,800
38	456	–	No_2	O-No_2 p-OCH_3	150-152	2430	–	2050	5300	9,200
57	471	OCH_2O	No_2	No_2		2280	–	2600	4220	15,600
26	457	CH_3O	No_2	No_2	228-229	2775	–	2070	–	15,290
48	426	No_2	No_2	–		2750	–	2500	4700	17,600
40	457	No_2	No_2	O-OCH_3	154-159	2760	–	4000	4900	10,100

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF CERTAIN TETRAZOLIUMS

Code No.	Biological Properties: Crystal Formation — Liver Cells 30'	Liver Cells 60'	Ascites Cells 30'	Ascites Cells 60'	DPNH Diaphorase System 10^{-9}M Formazan/Min.	Liver Cells ($5x10^5$/2ml) Endogenous Activity uM/$5x10^5$ cells	Liver Cells ($5x10^5$/2ml) Succinate Substrate uM/$5x10^5$ cells
None No_2							
4	0	0	0	0	0	0.000	0.066
8	0	0	0	0	0	0.000	0.083
5	0	0	0	0	0	0.000	0.087
17	0	0	0	0	0	0.000	0.118
6	0	0	0	0	0	0.000	0.046
44	0	0	0	0	0	0.000	0.061
54	0	0	0	0	0	0.000	0.073
45	0	0	0	0	0	0.000	0.286
Mono No_2							
49	0	0	0	$\pm$	48.9	0.058	1.090
42	0	$\pm$	0	+	15.8	0.052	0.471

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF CERTAIN TETRAZOLIUMS

Code No.	Biological Properties: Crystal Formation — Liver Cells 30'	Liver Cells 60'	Ascites Cells 30'	Ascites Cells 60'	DPNH Diaphorase System 10^{-9}M Formazan/Min.	Liver Cells ($5x10^5$/2ml) Endogenous Activity uM/$5x10^5$ cells	Succinate Substrate uM/$5x10^5$ cells
Mono No_2(cont)							
14	+	+	0	0	20.40	0.022	0.394
7	0	+	0	0	27.75	0.029	0.479
21	+	+	0	±	24.45	0.034	0.420
23	0	0	0	±	12.75	0.044	0.460
13	+	+	0	0	24.84	0.035	0.430
60	0	0	0	0	5.40	0.000	0.295
43	0	±	0	0	1.29	0.005	0.390
39	0	0	0	0	5.01	0.010	0.522
59	0	0	0	0	1.38	0.000	0.512
58	0	0	0	0	8.40	0.007	0.203
51	0	0	0	±	6.39	0.013	0.301

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF CERTAIN TETRAZOLIUMS

Biological Properties							
	Crystal Formation				DPNH Diaphorase System	Liver Cells ($5x10^5$/2ml)	
	Liver Cells		Ascites Cells			Endogenous Activity	Succinate Substrate
Code No.	30'	60'	30'	60'	10^{-9}M Formazan/Min.	uM/$5x10^5$ cells	uM/$5x10^5$ cells
Di-No_2							
50	0	0	0	+	106.2	0.174	0.510
38	0	0	0	0	76.8	0.158	0.760
57	0	0	0	+	81.0	0.071	0.318
26	0	0	0	±	36.3	0.137	0.496
48	0	0	0	0	4.41	0.153	0.440
40	0	±	0	±	33.3	0.109	0.317

Serial No. NCI-216
1. NCI
2. OADR
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Drug Development and Evaluation Program
Contract No. SA-43-ph-2371

Project Officer: A. Goldin

Assistant Project Officer: J. M. Venditti

Assistant Project Officer (Adm.): G. A. Brandner

Contractor: Microbiological Associates, Inc.

Sr. Investigator: I. Kline (1/2 time)

Full Time Personnel:

E. W. Artis	Crew Chief
H. Waters	Ass't Crew Chief
C. Jackmon	Sr. Technician
L. Keys	Sr. Technician
E. McCullough	Sr. Technician
G. Sanders	Sr. Technician
L. Mitchell	Sr. Technician
D. Jordan	Jr. Technician
W. Todd	Jr. Technician
M. Washington	Jr. Technician
L. Williams	Jr. Technician
J. Davis	Animal Caretaker
J. Keys	Animal Caretaker
J. McIlwain	Animal Caretaker
R. Wheeler	Animal Caretaker
S. Mangano	Statistical Clerk
J. Morse	Secretarial Service
E. Stevens	Janitor

Part Time Personnel (2/5 time):

C. Brown	Crew Chief
S. Sbitani	Sr. Technician
J. Exum	Jr. Technician
E. Phillip	Jr. Technician

C. Von-Der-Pool	Jr. Technician
H. Nicholls	Animal Caretaker
A. Stinson	Bacteriologist

Cooperating Units:

(a) Laboratory of Chemical Pharmacology, NCI: Serial #253

(b) General Medicine Branch, NCI: Serial #226

(c) Cancer Chemotherapy National Service Center, which supplied most of the compounds employed in these studies.

Man Years (calendar year 1960):

Total: 21 (adjusted for length of service and part time crew)
Professional: 2
Other: 19

Project Description:

Preclinical drug evaluation and development

Methods Employed:

Methods established for quantitative drug evaluation in the Laboratory of Chemical Pharmacology, NCI. Other applicable procedures. Standard pharmacological procedures.

Major Studies:

In accordance with the recommendations of the Director of Laboratories and Clinics, NIH; the Director, NCI; the Assistant Director (for Research), NCI; and the Clinical Director, NCI, the Drug Evaluation and Development Program has been in progress as part of the program of the Biochemical Pharmacology Section of the Laboratory of Chemical Pharmacology, NCI, for the further study of compounds of potential clinical interest. The studies conducted under this program have been directed to (a) the selection of drugs for clinical trial, (b) the obtaining of basic and practical information concerning the pharmacologic activity of such drugs, (c) the obtaining of information leading to the further study of compounds of interest at the biochemical level, and (d) the referral of specific clinical problems warranting animal experimentation. The program is being conducted by Dr. A. Goldin as Project Officer, J. M. Venditti as

Assistant Project Officer, and G. A. Brandner as Assistant Project Officer (Adm.) on contract with Microbiological Associates, Inc. The Project Officer is administratively responsible for the program to the Clinical Director, NCI; the Assistant Director (for Research), NCI; and the Director, NCI. Close liaison is maintained with the General Medicine Branch, NCI, the Laboratory of Chemical Pharmacology, NCI, and other groups in the intramural program. The program is run in close coordination with the Cancer Chemotherapy National Service Center which supplied most of the compounds employed.

The methodologies employed for the quantitative evaluation of compounds in experimental cancer have been developed primarily in the Laboratory of Chemical Pharmacology. Additional methods are being developed in the Laboratory of Chemical Pharmacology and in this program.

Three mouse tumors have been employed in the general evaluation of new agents of interest: Leukemia (L1210), sarcoma (S-37), and mammary adenocarcinoma (Ca-755). Limited studies have also been conducted employing the Ehrlich ascites tumor. Preliminary studies are being conducted on other experimental tumors including other solid tumors and virus induced tumors, in order to determine the feasibility of employing additional test systems in this program. In addition, certain compounds of interest have been subjected to detailed studies of the optimal schedules of treatment, the optimal route of administration, and the effects of treatment with combinations of drugs.

Increasing emphasis has been placed on studies of drug resistant tumors. These studies are being conducted in order to uncover drugs of potential clinical value in the treatment of refractory disease and to provide leads for the study of the mechanism of action of active compounds.

A. Studies with advanced leukemia L1210:

1. Employing amethopterin as a standard for antileukemic activity, 88 additional compounds were tested on subcutaneous treatment over a wide range of daily doses against advanced leukemia L1210. The compounds were rated relative to amethopterin with respect to their ability to increase the survival time of mice with advanced L1210. The structure-antileukemic activity relationships among these compounds may be summarized as follows:

Compound	Relative increase in median survival time over controls at the optimal daily dose. Amethopterin increase = 100
a. Alkylating agents	
(Of the alkylating agents tested up to this time in this program, Cytoxan has been the most effective, displaying about the same order of effectiveness as amethopterin.)	
[Bis(1-aziridinyl)phosphinyl] carbamic acid, ethyl ester: (AB-100)	59,49
Bis(1-aziridinyl)phosphonic acid, hexyl ester	58
1-Aziridineethanol, α-vinyl	43
Tetramin: (1-Aziridineethanol, α-vinyl mixed with 1-Aziridineethanol, β-vinyl)	30,27
p,p-Bis(1-aziridinyl)-N,N-dibutyl-phosphonic acid	21
Bis[2-(bis(2-chloroethyl)amino)ethyl]-sulfide·2HCl	19
Nor-nitrogen mustard	24,12,7
Epoxypropidine	19,18
1,3-Bis[bis(2-chloroethyl)amino] propane· HCl	17
2,5-Bis(1-aziridinyl)-3,6-dipropionamido-p-benzoquinone	16

5-Bis(2-chloroethyl)aminomethyl-4-(methoxymethyl)-2-methyl-3-pyridinol·2HCL·$2H_2O$	7
2-[Bis(2-chloroethyl)aminoethyl] benzimidazole	6
2,2'-Dichloro-2"-methoxy triethylene melamine	6
[Bis(1-aziridinyl)phosphinyl] carbamic acid, benzyl ester: (AB-103)	6
α-Chloropropyl-β',β"-dichloro-ethylamine·HCl	6
Di-β-chloroethylheptylamine·HCl	6
Diethyl-(β-chloroethyl)amine·HCl	6
2,2'-Dichloro-N-isopropyldiethylamine·HCl	6
N,N-Bis(2-chloroethyl)-2-dimethoxy aniline	6,0
1-Aziridineethanol, β-vinyl-	6,0
Quinacrine (ethyl) mustard	3
Chloroquine (ethyl phenyl) mustard	0
Quinacrine (propyl) half mustard	0
Bis(β-chloroethyl)-γ-naphthylmethyl amine·HCl	0
β-Chloroethyl piperidine·HCl	0
Chloroquine ethyl mustard	0
n-Propyl-ethyl-β-chloroethylamine	0
2,4-Diethyleneimino-6-methyl-5-nitro pyrimidine	0

Methanesulfonic acid, 2-chloro-, ethyl ester	0
Methanesulfonic acid, ethyl ester	0

b. Purine derivatives

(In general, 6-mercaptopurine was included in experiments involving the testing of purine derivatives. 6-Mercaptopurine dissolved in dilute NaOH has been, on the average, about 50 percent as effective as methopterin. It was found, however, that the activity of 6-mercaptopurine was somewhat reduced when it was suspended in methylcellulose.)

1-Methyl-1-nitroso urea	45, 16
2-Amino-6-(1'-methyl-4'-nitro-5'-imidazolyl) mercaptopurine	38,32,18
6-(1'Methyl-4'-nitro-5'-imidazolyl) mercaptopurine	22
6-Benzylthiopurine	19
9-Ethyl-6-mercaptopurine	18
Aminoguanidine sulfate	6
9-Butyl-6-mercaptopurine	3
9-Cyclopentyl-6-mercaptopurine	3
9-(2-Cyclohexenelyl)-6-purinethiol	0

c. Compounds with steriod activity

Prednisolone	19
Prednisone	16
9-α-Fluorohydrocortisone acetate	6

Cortisone	6
delta-testolactone	0,0
2-α-Methyldihydrotesterone propionic acid	0,0
Corticosterone	0
Diethylstilbestrol	0
Hexestrol	0
Hydrocortisone	0

d. Pyrimidine derivatives

2,4-Diamino-5-(3',4'-dichloro phenyl)-6-ethylpyrimidine	19
5-Mercaptouracil	6
5-Iododeoxyuracil	6,0
Orotic acid	0
6-Thiouric acid	0

e. Pyrazolo(3,4-d) pyrimidines

(Previous studies in this laboratory have shown that 4-aminopyrazolo(3,4-d) pyrimidine is 20 to 30 percent as effective as amethopterin.)

4-(3-Methoxypropylaminopyrazolo (3,4-d) pyrimidine	7
4-Isopentylamino-1-methylpyrazolo- (3,4-d) pyrimidine	7

f. Thiadiazole derivatives

(Previous studies in this laboratory have shown that the 2-amino and 2-ethylamino derivatives of

1,3,4-thiadiazole are 35 to 50 percent as effective as amethopterin.)

2-p-Toluenesulfonamide)-1,3,4-thiadiazole	9
2-Amino-5-methyl-1,3,4-thiadiazole	6
2-Amino-5-ethyl-1,3,4-thiadiazole	3
2-(p-Methoxybenzenesulfonamide)-1,3,4-thiadiazole	0
2-(Benzenesulfonamide)-1,3,4-thiadiazole	0

g. Pyridine derivatives

1-(β-Hydroxy-β-phenylethyl)-2-chloropyridinium bromide	0
β-Picoline	0
3-Pyridylmethylcarbinol	0

h. Nicotinic acid derivatives

N-Methylnicotinamide	6
N-Ethylnicotinamide	0

i. Podophyllotoxin derivatives

Podophyllotoxin isopropyl ammonium glutarate	10
Podophyllotoxin chloroacetate-ω-pyridinium chloride	8
Podophyllotoxin	0
Acetylpyridinium chloride podophyllotoxin	0
Picropodophyllin acetate	3,0
α-Peltatin	0

β-Peltatin	0

j. Others

4,6-Diamino-1-(3',4'-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine:(54.HCl)	12
Reserpine	12
Vincaleukoblastine sulfate	13,8
Ovamethidine	7,5
Singoserp	9,3
9-Chloro-9-(*p*-methylthioxyphenyl)-10-phenylacridan	6
4,6-Diamino-1-(4'-chlorophenyl)-1,2-dihydro-2-dimethyl-*s*-triazine	6
Leurosine	3
N-Dichloroacetyl-DL-serine (Na salt)	0
3-Hydroxy-2-phenylcinchoninic acid	0
Benzimidazole	0
Mucobromic acid	0
Quinonechloroimide	0
2-Ethylamino-4-diethylamino-5-chloro-*s*-triazine	0
Urethane	0

2. In addition to the 88 compounds tested against leukemia L1210 for the first time in this program within the last year, 16 selected compounds tested previously were reexamined in this assay system. The following table shows the degree of reproducibility of effectiveness found for these compounds.

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Part A - continued

Relative increase in median survival time over controls at optimal daily dose. Amethopterin increase = 100

Compound	Previous ratings	Current ratings
Nitrogen mustard	13,17,14	13
Alanine nitrogen mustard	12	14
N^4,N^4-Bis(2-chloroethyl)-N'-2-pyridylsulfanilamide	7	5
Triethylene melamine	32	32
Myleran	0	0
Nonane	23,7	5
2-Amino-1,3,4-thiadiazole	54	34
2-(p-Dimethylaminostyryl) quinoline methiodide	0,6	6
4-(p-Dimethylaminostyryl) quinoline,dihydrochloride	0	6
Glyoxalbisguanylhydrazone, monosulfate pentahydrate	9	0,6
Methylglyoxalbisguanylhydrazone,monosulfate, monohydrate	56	50,53
N-Acetylcolchinol	7	0
Colchinol	0	0
5-Diazouracil	0	3,0
Aminopterin	50,42,23,21	23
3'-Bromo-5'-chloroamethopterin	>413,339	428

3. Special Studies (advanced leukemia L1210):

In addition to the aforementioned comparisons of antileukemic effectiveness on daily subcutaneous treatment, further studies employing advanced leukemia L1210 were conducted as follows:

a. Route of administration. Previous studies showing that the antileukemic effectiveness of both 3',5'-dichloroamethopterin and amethopterin was diminished by oral administration of the compounds demonstrated the importance of studies of the influence of the route of administration on drug activity and suggested that greater emphasis be placed on such studies. It was shown that both 3'-bromo-5'-chloroamethopterin and 3'-bromoamethopterin were more effective when given subcutaneously than when given orally. Amethopterin was equally effective on subcutaneous, intraperitoneal, or intravenous administration, but again showed reduced activity when given orally.

Both triethylene melamine and nitrogen mustard were active when given either subcutaneously or intravenously, but were relatively ineffective on oral administration. Neither of these alkylating agents appeared to be more effective intravenously than subcutaneously, however. In addition, the subcutaneous activity of nor-nitrogen mustard was not enhanced by giving the compound intravenously.

Methylglyoxalbisguanylhydrazone, dihydrochloride monohydrate in two experiments showed activities of 34 and 59 percent subcutaneously and 22 and 44 percent orally. Methylglyoxalbisguanylhydrazone, monosulfate monohydrate displayed activities of 50 and 53 percent on subcutaneous administration and 28 and 34 percent when given orally. Of interest was the observation that glyoxalbisguanylhydrazone, monosulfate pentahydrate was not effective subcutaneously (0 and 6 percent) but showed definite but low activity on oral administration (12 and 16 percent).

The purine derivatives, 6-mercaptopurine, 6-thioguanine, and 2-amino-6-(1'-methyl-4'-nitro-5'-imidazolyl)mercaptopurine displayed about the same antileukemic activity when given subcutaneously or orally. 6-Azauracil displayed little or no antileukemic activity on either subcutaneous or oral administration.

b. Studies on the influence of the treatment schedule. The superiority of subcutaneous 3',5'-dichloroamethopterin over

oral administration of the compound against advanced leukemia L1210 obtained when treatments were given twice daily, daily, or once every fourth day.

6-Azauracil which was inactive on daily treatment showed no activity when the interval between treatments was decreased to 6 hours.

Vincaleukoblastine, which was about 10 percent as active as amethopterin on daily treatment, showed no appreciable change in activity when treatments were given every second day, every fourth day, or as a single treatment.

Epoxypropidine, which on daily treatment was 19 percent as effective as amethopterin, showed essentially unaltered activity when given every 3 days.

c. Studies of combination therapy. In continuation of studies of the treatment of systemic leukemia L1210 with combinations of Cytoxan and amethopterin it was shown that when each drug was employed on its optimal treatment schedule (treatment weekly for Cytoxan and treatment daily for amethopterin), combination therapy was superior to therapy with either drug alone.

Combination therapy employing urethane and amethopterin, urethane and nitrogen mustard, or urethane and Cytoxan was not more effective than treatment with amethopterin, nitrogen mustard, or Cytoxan alone.

Combination therapy with 6-azauracil and 6-mercaptopurine was not more effective than treatment with 6-mercaptopurine alone.

Reports of the potentiation of 5-fluorouracil (FU) and 5-fluorodeoxyuridine (FUDR) by 5-iododeoxyuridine (IUDR) prompted the investigation of various combinations of fluoronated pyrimidines in the advanced L1210 assay system. In confirmation of published reports, it was observed that concomitant treatment with IUDR enhanced the activity of suboptimal doses of both FU and FUDR. When comparisons between combination therapy and therapy with FU or FUDR alone were made at optimal dosage levels, however, treatment with IUDR plus FU or IUDR plus FUDR was found not to be more effective than treatment with FU or FUDR alone. Similar studies indicated that 5-mercaptouracil also failed to potentiate the maximum activity of FU.

d. Effect of structural isomerism on antileukemic effectiveness. The reported variability in the activity of Tetramin against a wide range of transplantable animal tumors and the recent clinical interest in this agent prompted its evaluation in the advanced L1210 assay system. In two experiments, the original sample of Tetramin was found to be 30 to 27 percent as active as amethopterin. Later it was learned that the original Tetramin represented a mixture of the secondary alcohol, α-vinyl-1-aziridineethanol with the primary alcohol, β-vinyl-1-aziridineethanol. Subsequent retesting of the isolated components showed that the secondary alcohol was 46 and 40 percent as effective as amethopterin while the primary alcohol was relatively ineffective. In toxicity studies in normal mice the primary alcohol showed toxicity for the host. The data indicate that the toxicity of the inactive component of the original Tetramin mixture rendered the mixture less effective than the secondary alcohol alone.

B. Studies employing drug resistant variants of leukemia L1210:

1. The 8-azaguanine resistant variant of leukemia L1210 (L1210/AgR).

a. This tumor which displays cross-resistance to 6-mercaptopurine was found to be resistant to 2-Amino-6-(1'-methyl-4"-nitro-5'-imidazolyl) mercaptopurine (ITG).

b. L1210/AgR was found to be sensitive to α-vinyl-1-aziridineethanol and to azaserine.

c. Studies of combination therapy with azaserine and 6-mercaptopurine confirmed the observation that the combination was more effective than either drug alone against advanced leukemia L1210, but was no more effective than azaserine alone against the subline L1210/AgR.

d. In general, studies with L1210/AgR, to date, have shown that this variant of leukemia L1210 is cross resistant to purine derivatives and partially resistant to pyrazolo(3,4-d)pyrimidines. Leukemia L1210 and the antipurine resistant subline, L1210/AgR display about equal sensitivity to alkylating agents and methylglyoxalbisguanylhydrazones. Amethopterin has been consistently more effective against the L1210/AgR subline than against leukemia L1210.

2. The amethopterin resistant subline of leukemia L1210 (L1210/M46R).

a. The following compounds displayed equal effectiveness against leukemia L1210 and the amethopterin resistant subline of L1210 (L1210/M46R):

Vincaleukoblastine

6-Mercaptopurine

Cytoxan

Methylglyoxalbisguanylhydrazone, dihydrochloride monohydrate

Uracil mustard

Thio-TEPA

4-Aminopyrazolo(3,4-d) pyrimidine

b. Partial or complete cross-resistance to L1210/M46R has been demonstrated for no compounds to date other than antifolics.

3. Studies with the L1210/C95 subline of leukemia L1210.

This variant of L1210 arose from successive treatment with amethopterin, 6-mercaptopurine, and Cytoxan over a number of transplant generations. L1210/C95 displays varying degrees of resistance to these compounds but shows no resistance to methylglyoxalbisguanylhydrazone as shown in the following table.

Compound	Percent increase in median survival time over controls	
	Adv. L1210	Adv. L1210/C95
6-Mercaptopurine	64	8
Amethopterin	127	23
Cytoxan	190	62
Methylglyoxalbis-guanylhydrazone	64	62

Preliminary studies employing other variants of leukemia L1210 are in progress. In the Laboratory of Chemical Pharmacology, other sublines of leukemia L1210 have been developed. The inclusion into this program of other resistant sublines as they may be developed is anticipated.

C. Studies with sarcoma (S-37):

1. Twenty-six compounds were studied for the first time with respect to their ability to extend the survival time of mice with relatively early S-37. Treatments were begun three days after tumor inoculation and given daily for 5 days. Each compound was administered over a wide range of doses and the median survival time at the optimal dose of each was compared with the median survival time of the untreated controls. The relative effectiveness of the compounds in increasing the survival time of the mice is shown in the following table.

Compound	Relative median survival time. Untreated controls = 1.0
p-N,N-Bis(2-iodoethyl)amino-benzyl phosphonic acid, ethyl ester	>3.0
Actinomycin D	1.4,1.2
Meta-Sarcolysin	1.3
N,N-Bis(2-chloroethyl)sulfanilic acid	1.3
A-139	1.3
L-Sarcolysin	1.2,1.1
3-Bis(2-chloroethyl)amino-tetrahydrofuran	1.1
N-Dichloroacetyl-DL-serine (Na salt)	1.2,1.1
Diethyl (β-chloroethyl) amine·HCl	1.1
9-(β-chloroethyl)carbazole	1.1
Nitromin	1.1

Compound	Relative median survival time. Untreated controls = 1.0
Chloroquine (ethyl phenyl)mustard	1.1
Epoxypropidine	1.1
Podophyllotoxin	1.1
Podophyllotoxin chloroacetate-ω-pyridinium chloride	1.1
Podophyllotoxin isopropylammonium glutarate	1.1
Vincaleukoblastine sulfate	1.1
2-[Bis(2-chloroethyl)aminoethyl]-benzimidazole·HCl	1.1,1.0
Bis[2-(bis(2-chloroethyl)amino) ethyl]-sulfide·2HCl	1.0,1.0
2,2'-Dichloro-N-isopropyldiethylamine	1.0
Tetramin (mixed isomers)	1.0
α-vinyl-1-aziridineethanol	1.0
β-vinyl-1-aziridineethanol	1.0
Nonane	1.0
2-[Bis(2-chloroethyl)amino methyl]-5,6-dimethyl benzimidazole·HCl	1.0
2,2'-Dichloro-2"-methoxy triethyl-amine·HCl	1.0

2. Special studies (S-37)

a. Influence of the treatment schedule. Studies of the influence of the duration of treatment on the effectiveness of compounds showed that Carzinophilin and 5-fluorouracil were more effective in extending the survival time of mice with early S-37 when given as a single treatment than when given daily for 5 days or daily to death.

Studies of the influence of the treatment schedule on the effectiveness of alanine nitrogen mustard against sarcoma 37 showed that daily treatment for 5 days was more effective than a single treatment. In addition, daily treatment of limited duration (5 days) was superior to continuous treatment to death employed on various schedules including every second day, every fourth day, and every seventh day.

b. Effect of caloric restriction on S-37. It was shown that caloric restriction had a moderate effect in inhibiting the local tumor growth of sarcoma 37. In general, mice in which there was appreciable tumor inhibition due to food restriction succumbed earlier than untreated mice receiving an ad libitum diet. A study was conducted in order to determine to what extent reduced food intake may account for the extension in survival time produced with N-methylformamide. The results showed that while treatment at an optimal dosage level with N-methylformamide substantially increased the lifespan of the mice, no level of food restriction produced an increase in survival time over the controls. Moreover, untreated mice receiving a diet isocaloric to the diet consumed by mice treated with N-methylformamide displayed little or no inhibition of local tumor growth and no increase in survival time.

c. Influence of the route of administration. Studies of the influence of the route of administration on the effectiveness of alanine nitrogen mustard on sarcoma 37 indicated that this compound was less effective when given intraperitoneally than when given subcutaneously.

The relative insensitivity of S-37 to therapy has precluded, to date, the use of the advanced form of this tumor in routine assay. Preliminary studies of advanced S-37 have shown that compounds which have the ability to increase the survival time of mice with early S-37 (e.g., N-methylformamide and alanine nitrogen mustard) have been inactive against more advanced stages of this tumor.

Sarcoma 37 is a tumor of interest in the drug development program because of the apparent lack of correlation between degree of tumor inhibition and increase in survival time attained as a result of therapy. For example, thioguanine, 6-mercaptopurine, Bayer 3231, and Actinomycin D have been shown to inhibit local tumor growth in mice with S-37, but none of these was able to increase the survival time of the mice.

D. Studies with advanced adenocarcinoma (Ca-755):

1. Previous studies conducted in this program employing Ca-755 had shown that a substantial number of compounds including many purine derivatives, Cytoxan, 5-Fluorouracil and its riboside, and Mitomycin C increased the survival time of the mice when treatment was begun early in the course of the disease (day 4). During the past year, the treatment of more advanced Ca-755 has been emphasized. Treatment over a wide range of doses was initiated when the local tumors had achieved a diameter of at least 10 mm. Two schedules of treatment have been routinely employed. In one the duration of daily treatment was limited to 5 days. In the other, treatment was given daily until death. The relative effectiveness of 40 compounds tested for their ability to extend the survival time of the mice with advanced Ca-755 is summarized as follows:

Compound	Relative median survival time Controls = 1.0	
	Daily treatment for 5 days	Daily treatment to death
(1) Purine derivatives		
6-Mercaptopurine	1.3 to 2.3*	1.5 to 1.9*
6-Mercaptopurine riboside	3.2,1.5	1.2
6-Chloropurine	1.6	1.5
9-Butyl-6-mercaptopurine	1.3	
6-(sec-Butylthio)purine	1.3	
2-Amino-(6-benzylthio)purine	1.3	1.1
6-(Isopropylthio)purine	1.3	
6-(Benzylthio)purine	1.2	1.2
6-(Isobutylthio)purine	1.2	
9-Ethyl-6-mercaptopurine	1.2	

* Range for 20 experiments

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Compound	Relative median survival time Controls = 1.0 Daily treatment for 5 days	Daily treatment to death
6-Thioguanine	1.2	1.3
8-Azaguanine	1.2	
6-Thioguanine riboside	1.1	1.1
2-Amino-6-(1'-methyl-4'-nitro-5'-imidazolyl)-6-mercaptopurine	1.1	
9-Cyclopentyl-6-mercaptopurine	1.1	
6-(n-Octylthio)purine	1.0	
6-(Allythio)purine		1.5
6-(n-Butylthio)purine		1.3
(2) Alkylating agents		
Cytoxan	1.9	1.5
Glycine nitrogen mustard	1.1	1.0
Nitrogen mustard	1.1,1.0	1.0
Alanine nitrogen mustard	1.0	1.0
(3) Antibiotics		
Actinomycin D	1.3	
Streptovitacin A	1.1	1.1
Mitomycin C	1.1	1.1
Chloramphenicol	1.1	
Carzinophilin	1.0	1.1

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Compound	Relative median survival time Controls = 1.0	
	Daily treatment for 5 days	Daily treatment to death
(4) Pyrimidine derivatives		
5-Fluorodeoxyuridine	1.1	1.1
6-Azauracil	1.0	
(5) Pyrazolo pyrimidines		
4-Aminopyrazolo(3,4-d) pyrimidine	1.4	1.2
4-Isopentylamino-1-methyl-pyrazolo(3,4-d)pyrimidine	1.2	1.1
(6) Podophyllotoxin derivatives		
Podophyllotoxin chloro-acetate-4'- pyridinium chloride	1.2	
Podophyllotoxin	1.1	
Podophyllotoxin isopropyl ammonium glutarate	1.1	
(7) Others		
Vincaleukoblastine sulfate	1.3	
6-Azaserine	1.1,1.1	
N-Methyl-N-nitro-N-nitroso-guanidine	1.1	
Diethylstilbestrol	1.1	
Aminoguanidine sulfate	1.0	
1-Methyl-1-nitroso urea	1.0	

2. Special studies with advanced adenocarcinoma (Ca-755).

a. Influence of the schedule of treatment. 6-Mercaptopurine was found to be more effective against Ca-755 when given every second day than when given twice daily, daily, every fourth day, or as a single treatment.

b. Influence of the route of administration. Studies of the influence of the route of administration on the effectiveness of treatment of Ca-755 showed that neither 6-mercaptopurine nor 2-amino-6-(1'-methyl-4'-nitro-5'-imidazolyl)mercaptopurine was more effective when given orally than when given subcutaneously.

c. Effects of combination treatment. Studies of combination treatment of Ca-755 with azaserine and 6-mercaptopurine indicated that the combination was not more effective than 6-mercaptopurine alone in extending the survival time of the mice. Azaserine alone provided little increase in survival time. This is in contrast to the results of the treatment of advanced leukemia L1210 with a combination of 6-mercaptopurine and azaserine. With advanced leukemia, both compounds increased the survival time of the mice, 6-mercaptopurine being the more effective. Also, against advanced leukemia L1210, the combination was more effective than treatment with either drug alone under appropriate dosage schedules. Combination treatment of Ca-755 with 6-azauracil and 6-mercaptopurine was not more effective than treatment with 6-mercaptopurine alone.

d. Effect of caloric restriction. It was shown that caloric restriction was able to both inhibit local tumor and increase the survival time of mice with advanced Ca-755. The degree of tumor inhibition or increase in survival time attained by food restriction was less than that provided by treatment with 6-mercaptopurine in the same experiment.

E. Studies with the Ehrlich ascites tumor:

Eight compounds were investigated for their ability to increase the survival time of mice bearing this tumor. Daily subcutaneous treatment over a wide range of doses was begun 4 days after tumor inoculation and continued until death. At the optimal daily dose, N-methylformamide provided an increase in median survival time of from 40 percent over untreated controls. 6-Mercaptopurine, amethopterin, Cytoxan, nitrogen mustard, DL-sarcolysin, glycine nitrogen mustard, and alanine nitrogen mustard failed to increase the survival time of the mice.

Significance to the Program of the National Cancer Institute:

Two factors which have limited the success of chemotherapy have been (a) the toxicity of the drug for the host and (b) the capacity of the tumor to develop resistance to therapy.

Relative to the former problem, the Drug Development and Evaluation program is devoted primarily to determining quantitatively the relative antitumor specificity of drugs of potential clinical interest. In particular, the program emphasizes structure-activity relationships among compounds which emerge as "active" from primary screens. In the Drug Development and Evaluation Program, prolongation of the lifetime of tumor bearing animals is employed as the chief criterion for determining the relative therapeutic efficacy of drugs. Tumor size and body weight changes are, nevertheless, measured routinely. The program also investigates "active" compounds with respect to the optimal conditions for antitumor activity including the optimal schedule of treatment, route of administration, effect of combination therapy, etc. Such pharmacologic studies are needed to provide an accurate appraisal of the potential usefulness of a compound of interest.

Studies of the problem of the development of resistance to treatment have been facilitated by the emergence of active compounds of various chemical classes which have the capacity to inhibit growth at different biochemical sites and by the development of drug resistant sublines of tumors. The inclusion of studies of the effects of new compounds on drug resistant tumors is valuable from two standpoints. Such studies can uncover active compounds which may have clinical usefulness in cases which have become refractory to conventional treatment. In addition, drug resistant tumors are being utilized to explore possible mechanisms of drug action.

The various areas of study included in the Drug Development and Evaluation Program and the collaboration with cooperating units provide a means for helping in the selection of compounds for clinical trial. The program provides an avenue for the quantitative evaluation of compounds found"active" in the primary screen sponsored by the Cancer Chemotherapy National Service Center. The close collaboration between the Drug Development and Evaluation Program and the General Medicine Branch of the NCI facilitates the investigation of clinical problems at the experimental animal level. The Drug

Development and Evaluation Program provides the framework for a well-integrated program devoted to the study of compounds of clinical interest at the biological, pharmacological, and biochemical levels.

Proposed Course of Project:

During the next year, the various aspects of this program will be continued and expanded to include other test systems which may be appropriate. The possibility of the inclusion of assay systems employing virus induced tumors is being investigated. An assay system employing the Moloney whole cell virus leukemia has been developed in the Biochemical Pharmacology Section of the Laboratory of Chemical Pharmacology, in collaboration with Dr. John Moloney, Laboratory of Biology, NCI. It appears suitable for incorporation into this program.

In the various test systems used, structure-activity relationships among compounds will be emphasized. The program will continue to give particular attention to factors which may alter the host-tumor-drug relationship. Increasing attention will be given to the problems of the development, prevention, and treatment of resistance. In this regard, therapeutic testing will emphasize studies with drug resistant tumors and studies of the effects of combination therapy.

Part B included: Yes

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Venditti, J. M., Goldin, A., and Kline, I.: The Influence of Treatment Schedule on the Chemotherapy of Advanced Leukemia L1210 in Mice. Cancer Chemotherapy Reports 6, 55-57, 1960.

(2) Goldin, A., Venditti, J. M., Kline, I., and Mantel, N.: Evaluation of Antileukemic Agents Employing Advanced Leukemia L1210 in Mice. II. Cancer Research, Cancer Chemotherapy Screening Data VI, 20, 382-448, 1960.

(3) Venditti, J. M., Humphreys, S. R., Mantel, N., Kline, I., and Goldin, A.: Evaluation of Antileukemic Agents Employing Advanced Leukemia L1210 in Mice. III. Congeners of Folic Acid. Cancer Research, Cancer Chemotherapy Screening Data VIII, 20, 698-733, 1960.

(4) Venditti, J. M., Schrecker, A. W., Mead, J.A.R., Kline, I., and Goldin, A.: Influence of the Route of Administration on the Relative Effectiveness of 3',5'-Dichloroamethopterin and Amethopterin Against Advanced Leukemia (L1210) in Mice. Cancer Research 20, 1451-1456, 1960.

(5) Venditti, J. M., Frei, E. III, and Goldin, A.: The Effectiveness of 2-Amino-6-(1'-methyl-4'-nitro-5'-imidazolyl) mercaptopurine against Transplantable Mouse Leukemia. Cancer Chemotherapy Reports 8, 44-46, 1960.

(6) Goldin, A., Venditti, J. M., Kline, I., and Mantel, N.: Evaluation of Antileukemic Agents Employing Advanced Leukemia L1210 in Mice. IV. In press, Cancer Research, Cancer Chemotherapy Screening Data.

Additional publications, other than abstracts, from the Biochemical Pharmacology Section, Laboratory of Chemical Pharmacology, NCI. (See Project Report NCI-253).

Honors and Awards relating to this project:

See Project Report NCI-253

Serial No. NCI-203 (a, b)
1. OADR
2. Environmental Cancer
3. Bethesda, Md.

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Part A

Project Title: - Macromolecular Polymers as Carcinogenic Agents

a. Experimental Studies on Water Soluble Macromolecules: Bioassay on Polyvinylpyrrolidones with Limited Molecular Weight Range

Principal Investigator - W. C. Hueper

b. Carcinogenic Studies on Water-Insoluble Polymers

Principal Investigator - W. C. Hueper

Man Years (calendar year 1960)

Total	0.5
Professional	0.1
Other	0.4

a. Bioassay on Polyvinylpyrrolidones with Limited Molecular Weight Range

Objectives: - - Previously reported investigations on several water soluble macromolecular substances (PVP, dextran, PVA, MC, starch) of different average molecular weight and varying molecular sizes and shapes had demonstrated an inconsistency of carcinogenic reactions in rats, mice and rabbits which could not be related to the variations of the physical factors cited, and peculiar to the different macromolecular materials tested. Since all macromolecular chemicals so far employed contained a fraction of sufficiently high molecular weight and size which prevented its passage through biologic filter membranes and thereby caused its retention in the tissues of various organs, the possibility existed that the development of cancerous responses might be related to the local storage of these substances. This concept could be put to a test when two polyvinylpyrrolidones (PVP) of limited molecular weight range became

a - (cont)

available, one of which had a maximal molecular weight of 38,000 while the second one had one of up to 80,000. It could be expected, therefore, that the first PVP could totally be excreted, whereas the second one would in part be retained.

Methods Employed: - - The two polyvinylpyrrolidones were furnished as 25% (w/v) neutral aqueous sterilized solutions in flasks each containing 100 cc. of a slightly yellow, viscous liquid. Kollidon K17 consisted of macromolecules having a molecular weight between about 2,000 and a maximum of about 38,000 with the bulk of the molecules having a weight between 5,000 and 15,000 (peak at 10,000). Kollidon K25 was composed of molecules the majority of which had a molecular weight of 15,000 to 30,000 (peak at 18,000), although a not inappreciable fraction had weights up to 80,000 (table 1). Active oxygen from the catalyst used was demonstrable in K17 to the amount of 0.05% expressed as hydrogen peroxide and in K25 to the amount of 0.005%.

For comparative purposes, two polyvinylpyrrolidones (#6 and #7), one of American make and the second of German manufacture which had been used in a previous experiment in the form of subcutaneous implants of dry powder, were employed this time as 20% normal saline solutions. Both PVPs had an average molecular weight of 50,000, PVP#7 had displayed a weak carcinogenic effect upon repeated subcutaneous implantations.

With the observation of sterile techniques the solutions of the four PVPs were administered by injection into the abdominal cavity of rats and rabbits. For testing the PVPs K17 and K25 sets of 35 female rats of the Bethesda black strain, about 3 months old, and of 6 rabbits of the Dutch strain of about the same age were used. The rats received four intraperitoneal injections of 2 cc. each at intervals of 2 weeks, containing a total of 2 gms. of the PVP. The rabbits weighing about 2,500 gms. were given by the same route, individual doses of 50 cc. up to a total of 5 injections delivered at 2-week-intervals, containing a total of 62.2 gms. of PVP.

PVP 6 (General Aniline and Film Corporation (Plasdone C)) was injected as a 20% solution in saline into 20 female Bethesda black rats, while PVP 7 (Schenley Laboratories PVP PDR) was administered to 30 rats. Each rat was given intraperitoneally at each injection, 5 cc. The injections

a - (cont)

were made 3 times a week for 5 weeks until a total of 9 injections had been reached or a total of 9 gms. of the PVP had been introduced into each rat. The further administration of the PVP solutions which originally had been planned to encompass 15 injections, was temporarily discontinued after the ninth injection because of the development of progressive and severe loss of weight of the treated animals. The injections were, however, resumed one month later after the rats had recovered from the initial untoward effects until a total of 15 injections had been made and thus a total of 15 gms. of the PVP had been introduced into each animal.

Two control rabbits received in place of the PVP solutions, intra-abdominal injections with physiological saline in amounts of 50 cc. each at intervals of 2 weeks and until a total of 250 cc. had been given. Thirty Bethesda black rats likewise were injected intraperitoneally with 2 cc. of normal saline (0.85% NaCl) for a total of 4 injections given at intervals of 2 weeks.

Blood studies were performed on the rabbits which received PVP injections. The erythrocytes, leucocytes were counted in 2-week-intervals before each injection. A differential count of the white cells was made. After each injection the bleeding time, coagulation time and the sedimentation rate were determined at intervals of 1, 2, and 3 hours after each injection.

Major Findings: - -A prominent finding made during the histologic examination of the tissues of rats and rabbits injected with the four PVPs was the presence of light-blue, deep-blue- or slate-blue-stained homogeneous matter within foam cells as well as in places also in the form of extracellularly located "lakes" in various tissues, such as the lung, heart, aorta, pulmonary artery, spleen, pancreas, choroid plexus, ovary, uterus, adrenal, liver, kidney, and the stroma of some cancers. The organs and tissues of the control animals were totally free from such deposits. They were also absent in the tissues of the rabbits receiving solutions of PVP K17, but were well developed in those injected with PVP K25. The only histologically demonstrable findings suggesting in the rabbits injected with PVP K17 solutions that a transitory macromolecular colloidal disturbance of the blood had occurred was presented by the small fibrous intimal cushions in the aorta and in the branches of the pulmonary artery in two rabbits. The various types of cancers observed in the four series of animals injected with PVPs were identical in location and in structure with those seen in the control rats. In none of the test series were cancers more

Table 1 --Death distribution of rats and rabbits injected with polyvinylpyrrolidone and number, sites and types of cancers.

Agent	Species	No.	Months of survival after start								No. cancer
			0-6	7-9	10-12	13-15	16-18	19-21	22-24	25-28	
PVP K17	Rat	35			3 1b	7 1b	9	4 1a	12 1a, 1b		5
	Rabbit	6			1				2	3	0
PVP K25	Rat	35	5	2	8	7	5	3 2a, 1c	5		3
	Rabbit	6	2						1	3	0
PVP #6	Rat	20	5	1	3	1 1b, 1d	5	2	3		2
PVP#7	Rat	30	2	1	7	2	2	11	5		0
Control	Rat	30	2	1	5 1b	2	5	6 1a, 1b	9 2a		5
	Rabbit	2									0

a -- Carcinoma of the uterus.
b -- Reticulum cell sarcoma of the liver.
c -- Round cell sarcoma of the ileocecal lymph nodes.
d -- Round cell sarcoma of the subcutaneous tissue.

Major Findings (cont):

frequent than in the control series. In fact, cancers were not observed among the rats which received PVP #7.

Significance: - - The macromolecular material of both PVPs was retained in the organs of rats despite the relatively low maximal molecular weight of one of the PVPs. However, this PVP passed the glomerular filter of the rabbits and thus was not stored.

The glomerular filter of rats and rabbits seems to differ in pore size.

The degree of storage of the PVPs tested did not exert any stimulating effect upon the production of cancers. In fact, there was evidence that one of two PVPs used for comparative purposes and having an average molecular weight of 50,000 might have interfered with the development of spontaneous cancers.

Carcinogenic properties of PVPs, whenever they are present do not seem to be related to any physical properties of the macromolecules.

Proposed Course: - - These studies have been terminated for the time being since their profitable continuation would require the active cooperation of a physical chemist.

PART B Included: - YES X

PART B:

Publications: - - Hueper, W. C., Bioassay on Polyvinylpyrrolidones with Limited Molecular Weight Range: J. National Cancer Institute, in press.

Serial No. NCI-203 (b)
Calendar Year 1960

b. Carcinogenic Studies on Water Insoluble Polymers

Objectives: - - The rapidly increasing use of synthetic polymers in industry as well as in medicine poses an important question concerning their ultimate safety from a carcinogenic viewpoint. In a recently published article of Harris on this subject, the statement is made that "probably the most decisive evidence of the safety of the use of selected plastics in contact with food and consumable liquids is provided by the large number of successful implantations in human tissues for surgical purposes". He, unfortunately, omits any consideration of the rather numerous observations on the carcinogenic reactions elicited by plastic implants of various chemical composition (polyethylene, cellophane, polyacrylates, polyvinyl compounds, polysilicones, etc.) in rats and mice (Oppenheimer and Associates; Hueper; Oettel). Since all of the cancerous responses so far obtained to such agents were sarcomas, their potential importance for man has been minimized by some investigators by the assertion that rats show an abnormal sarcomatogenic reactivity of their connective tissue to parenterally introduced foreign bodies of nonspecific chemical nature (Oettel; Nothdurft) and that for this reason these "sarcomas" occupied a special position(Brunner). In short, the observations made in rats on the carcinogenic action of plastics were allegedly not applicable to and without significance to man.

Nothdurft; Oppenheimer et al.; Shubik; and others, moreover, have advanced the concept that the causative mechanism active in the production of "polymer cancers" is not of chemical nature but represents a new carcinogenic principle related to some unidentified and mysterious surface forces possessed especially by polymer films and that, in fact, the carcinogenic effect of polymer implants decreases and finally disappears with a progressive reduction of an uninterrupted surface of polymer implants, i. e., powdered polymers are allegedly devoid of carcinogenic properties.

Because of the important influence which the acceptance of these scientifically ill-founded concepts might exert upon the continued use of such agents in industry and medical practice, three chemically different polymers in various physical states were implanted into the subcutaneous tissue and into the peritoneal cavity of rats so as to test the validity of the allegation that observations on polymer carcinogenesis had destroyed the long-accepted concept of chemical carcinogenesis and that the concept of specific carcinogenic chemicals had become obsolete and scientifically unsound (Nothdurft).

Methods Employed: - - Three polymers were selected for the study,

Methods Employed:(cont)

namely, polyethylene, polyurethane and polysilicone. Polyethylene, essentially a paraffinic, long-chained and usually somewhat branched carbon polymer was implanted in the form of cubes, round discs, film, and powder in amounts of equal weights. Polysilicone was used as rubber and gum, while polyurethane was employed in the form of cubes, foam and powder. Polysilicone rubber and gum were implanted also into the subcutaneous tissue and into the abdominal cavity of C 57 black mice. Sets of 30 rats and 50 mice were used for each experiment.

Major Findings: - - The three types of polymers used, elicited cancerous reactions in the tissues surrounding the implants. The degree of carcinogenic response varied for each polymer with the site of implantation and with its physical state (table 1).

Table 1 - - Physical state of implant, carcinogenic response at site of deposition and latent period of polymer cancers in rats.

Physical State	Agent	Carcinogenic Response at Site		Latent Period in Months
		Intraperitoneal	Subcutaneous	
Cube	Polyethylene	2		13
	Silastic Rubber	0	10	10
	Polysilicone Gum	0	1	5
Sheet-Disc	Polyethylene	1		22
	Polyurethane	0	1	22
Film	Polyethylene	6		16
Foam	Polyurethane	9	1	10
Powder	Polyethylene	0		
	Silica	0	0	
	Polyurethane	1	0	16

Significance: - -

While the cancer yield obtained with polyethylene in its four different physical states seems to support in a general way the concept of the cancerigenic importance of the size of the uninterrupted surface area of a polymer, the observations made in this respect with the other two polymers are in stark disagreement with this concept. In fact, the by-far-highest yield of cancers produced by polyurethane plastic is related to polyurethane foam in which the surface contains innumerable small perforations. It is noteworthy, moreover, that polyurethane powder was as effective in cancerigenic respects as polyurethane discs, i. e., a form of implant which for reasons of surface area has figured prominently in the argumentation of

Significance : (cont) --

Nothdurft as the carcinogenically most effective type of polymer implant. It is also worth mentioning that the smooth surfaced polysilicone gum implants were distinctly less carcinogenic than the rather rough surfaced cubes of the polysilicone rubber (Silastic). The concept of the importance of a smooth surface in carcinogenic respects, emphasized by Nothdurft, breaks down completely when the considerable carcinogenic action displayed by polyurethane foam is considered. In fact, the tissue reaction to polyurethane foam is especially remarkable since there is no real capsule formation but a complete penetration of the foam by connective tissue with the formation of a multicystic foreign body giant-cell granuloma which ultimately encloses small scaly fragments of the disintegrated foam. The cancer formation related to polyurethane foam is thus associated with a tissue reaction which Nothdurft describes as present for polymer powders and to which he attributes the lack of cancer formation from such materials. Powdered polyurethane foam brought into the abdominal cavity of rats, produced one definite polymer cancer and possibly a second one. From the wide distribution of foreign body granulomas in the abdominal cavity of rats given polyethylene and polyurethane in powdered form, it is obvious that neither the uninterrupted surface nor the lack of capsule formation but the evidently small amounts of active material at any particular site is the carcinogenically most important factor.

It is evident from these observations and considerations that purely physical factors as represented by a large uninterrupted and smooth surface as well as capsule formation and mechanical pressure play scarcely a significant or essential role in polymer carcinogenesis.

This interpretation of the evidence obtained receives additional support from the following findings. Despite the fact that the weight of each type of polymer was identical, the highest tumor yield was seen with that physical state which permitted the most intimate contact with the polymer, i. e., film or foam, at one single site. It may be pointed out that high carcinogenic potency of Silastic rubber which seems to represent an exception to this rule, can be explained by the fact that the rubber is actually a foam of vulcanized polysilicone gum whose meshes are occupied by the filler, i. e., silica. In this important aspect silastic is comparable to polyurethane foam. The probability of a chemical interaction between implant and body cells surrounding it as an important factor in polymer carcinogenesis as suggested by these findings, is further supported by the demonstration of the destructive effect of body fluids and cells upon

Significance: (cont)

polyurethane foam and of the polymer fragments produced thereby within the cancer tissue. The action of a chemical mechanism is also indicated by the development of adenocarcinomas from the cecal mucosa, although the polyurethane foam cushion was merely adherent to the serosal surface of this organ. No known physical carcinogen except ionizing radiation exists, according to presently available information, which might elicit cancers at such a distance from the carcinogenically reacting tissue. Chemical carcinogens, on the other hand, would readily account for such phenomena, particularly since the cancers elicited at this site were carcinomas and not sarcomas. The exact nature of the carcinogenic principle responsible for these cancers, however, remains to be determined. Although some aspects of polymer carcinogenesis have remained obscure and in need of further elucidation, the evidence presented does not support the concept of a physical mechanism of polymer cancers but rather favors the action of a chemical mechanism. The phenomenon of polymer carcinogenesis cannot be regarded as a nonspecific reaction to a parenterally introduced foreign body possessing certain specific physical properties. Polymer cancers, therefore, do not provide any sound scientific reason for discarding as obsolete the concept of "specific chemical carcinogens" as advocated by Nothdurft.

Proposed Course: - - Because of the marked variations in the type and number of chemical additives present in polyurethane foams and their extensive use in orthopedic and cosmetic surgery, three different polyurethanes (polyester of polyether products) have been procured and are being used in implants for ascertaining whether carcinogenic properties of polyurethane foams depend upon the polymer proper or on the presence of some specific additive in the polymer. One polyester type and one polyether type of polyurethane are, moreover, being fed to rats.

Since it is somewhat uncertain whether completely vulcanized Silastic (rubber) was used in the previous experiments, an additional group of rats has been implanted with discs of Silastic rubber for confirming the cancerous results obtained.

Because it has been claimed that the catalyst, benzoyl peroxide, used in the manufacture of Silastic is the carcinogenic agent, 30 rats have received subcutaneous implants into the nape of the neck for testing this concept.

PART B INCLUDED : - - YES

Serial No. NCI-203(b)
Calendar Year 1960

PART B:

Publications: - - Hueper, W. C., Experimental Production of Cancers by Implanted Polyurethane Plastic. Am. J. Clin. Path. 34:328-333, 1960.

Hueper, W. C., Carcinogenic Studies on Water-Insoluble Polymers. Pathologia and Microbiologia, in press.

Serial No. NCI-204 (c)
1. OADR
2. Environmental Cancer
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: - Carcinogenic Studies on Petroleum Substitutes and Synthetic Liquid Fuels

c. Carcinogenic Studies on Road- and Roofing-Asphalts(Petroleum)

Principal Investigator - - W. C. Hueper
Other Investigator - - - - Wm. W. Payne

Man Years (calendar year 1960)
Total : ---------- 0.1
Professional: ----- 0
Other: ---------- 0.1

c. See detailed Report for Calendar Year 1959; Project Terminated 1959

PART B INCLUDED: - - YES.

Serial No. NCI-204(c)
Calendar Year 1960

PART B:

Publications: - - Hueper, W. C., and Payne, W. W., Carcinogenic Studies on Petroleum Asphalt, Cooling Oil, and Coal Tar. Arch. Pathol. 70:372-384, 1960.

Serial No. NCI-205 (a-1, a-2,
b-1, b-2,
b-3, b-5)

1. OADR
2. Environmental Cancer
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A;

Project Title: - Experimental Studies in Metal Cancerigenesis

a. Experimental Studies on Cancerigenic Properties of Metallic Nickel:

1. Prolonged Inhalation of Powdered Metallic Nickel in Combination with Small Concentration of Sulfur Dioxide by Guinea Pigs, Rats and Hamsters.

Principal Investigator - - W. C. Hueper
Other Investigator - -- Wm. W. Payne

2. Intrapulmonary Injection of Nickel Suspensions in Gelatin Solution into Rats.

Principal Investigator - W. C. Hueper

b. Biologic and Biochemical Studies on the Cancerigenic Action of Chromium and Chromium Compounds:

1. Intrapleural, Intramuscular and Intratracheal Injections of Chromite Ore Roast and of Several Pure Chromium Compounds

Principal Investigator - W. C. Hueper
Other Investigator - - Wm. W. Payne

2. Chronic Inhalation of a Mixture of Chromite Ore and Chromite Ore Roast with Limestone Dust and its Effect on the Respiratory Organs of Mice, Rats, Guinea Pigs and Rabbits.

Principal Investigator - - W. C. Hueper
Other Investigator - - - Wm. W. Payne

Part A: (Cont.)

b-3: Characterization and Bioassay of Dust from a Chromate-Producing Plant.

Principal Investigator - Wm. W. Payne
Other Investigator - - - W. C. Hueper (Pathology).

b-4: (Terminated)

b-5: Prolonged Inhalation of Chromic Oxide and of Zinc Chromate, Barium Chromate and Lead Chromate by Rats.

Principal Investigator - - W. C. Hueper
Other Investigator - - Wm. W. Payne

Man Years (Calendar year 1960)
Total: -------- 2.1
Professional: ---- 0.4
Other: -------- 1.7

a-1: - Prolonged Inhalation of Powdered Metallic Nickel in Combination with Small Amounts of Sulfur Dioxide by Guinea Pigs, Rats & Hamsters.

This project has been reported in detail for the calendar year 1959.

Major Findings: - - Two hamsters showed at autopsy large-to-medium-sized-tumors of the adrenal gland, while two additional ones had large tumor masses involving the ileocecal lymph nodes. None of the hamsters had any pulmonary lesions suspect of cancerous nature. Five of the rats, on the other hand, revealed white medullary masses affecting the lungs.

Proposed Course: - The experiments will be continued until a maximal exposure period of two years has been reached. Exposure has now been completed. The results will be evaluated as soon as histologic examination has been completed. The experiments with nickel will then be terminated.

PART B INCLUDED: - - NO

Part B Included: NO

a-2. Intrapulmonary Injection of Nickel Suspensions in Gelatin Solution in Rats.

This project has been reported in detail for the calendar year 1959.

Major Findings: - - Of the 27 rats used, 15 have died. One of them revealed a tumor mass in the injected lung.

Proposed Course: - -The rats will be kept under observation for an additional year.

PART B - NO

b-1. Intrapleural, Intramuscular and Intratracheal Injection of Chromite Ore Roast and of Several Pure Chromium Compounds

Objectives: - Despite the demonstration of a serious lung cancer hazard for manufacturers of chromates in Germany, the U nited States and Great Britain, all attempts made by several investigators to produce such cancers in animals by the use of various experimental procedures and chromium compounds had failed or given equivocal results. Since it appeared possible that these failures might be attributable to the fact that substances were used which either were not sufficiently soluble in a watery physiologic medium for permitting a release of adequate amounts of biologically active chromium (chromium metal, chromite ore) or were too corrosive and therefore exerting an exclusively necrotizing effect upon the cells upon contact, chromite ore roast was tested because it contains in addition to sodium monochromate and chromite ore, various intermediary stages of oxidized chromium compounds as well as chromium polymers being of moderate solubility and lacking acute necrotizing qualities.

The apparent validity of these concepts was attested to by some preliminary experimental results obtained in rats which received intrapleural and intramuscular implants of chromite ore roast suspended in sheep fat. This material contains various chromium compounds differing markedly in their solubility in water.

Methods Employed: - - Chromite ore roast, an intermediate product in the production of chromium chemicals from

b-1 (Cont):

chromite ore, is either obtained by the roasting of chromite ore only (primary roast) or represents the product of roasting (secondary roast). Since, in the original experiment, a mixture of the two roasts was used, it was important to determine whether the two types of roast might vary in carcinogenic potency, because they could be expected to differ in the quantitative and qualitative composition of chromium compounds. Two sets of 35 rats each received implants of 25 mg. of the primary roast residue (most of the sodium chromate removed from roast by leaching at the plant) into the pleural cavity and into the muscle tissue of the right thigh, respectively.

Because of the partly uncertain and varying nature of the chromium compounds in chromite ore roast residue, additional experiments were set up using pure (above 99%) chromium compounds differing among each other in their relative solubility in water, some of which possibly were formed during the roasting process, others extensively employed in the human economy as pigments and anticorrosives (calcium chromate, sintered calcium chromate, magnesium chromate, sintered magnesium chromate, strontium chromate, zinc chromate, barium chromate, lead chromate and sintered chromium trioxide or chromic chromate(Cr_5O_{12})). As in previous experiments, implants containing 25 mg. and suspended in sheep fat, were introduced into the muscle tissue of the femur and into the pleural cavity, respectively, of sets of 35 rats each. These experimental approaches have recently been supplemented by the repeated monthly intratracheal injection of 0.05 cc. of a 20% solution of gelatin containing about 2 mg. of the particular chromium compound (calcium chromate).

The original experimental approaches have been supplemented by:

1. Intrapleural and intramuscular implantation of chromic acetate in gelatin capsules in rats.

2. Intratracheal administration of calcium chromate in gelatin solution to rats.

3. Intrapleural and intramuscular injections of sodium dichromate solution to rats at monthly intervals.

b-1 (cont).

4. Intratracheal administration of strontium chromate in gelatin solution to rats.

These supplements to the original experiment were undertaken to try to produce carcinoma of the lung, to ascertain whether trivalent chromium compounds will also produce tumors in animals and to determine whether the repeated administration of a highly soluble chromium compound will produce the same results as a depot of slightly soluble compounds.

Major Findings: - - The results are summarized in Tables 4, 5 and 6 of the article entitled, "Experimental Studies on Chromium Compounds, Their Carcinogenicity and Their Importance for Industrial Medicine" which was presented at the 13th International Congress on Occupational Health, New York City, July 28, 1960, which will be published in the Proceedings of this Congress.

Significance: - - The prolonged inhalation of powdered chromite ore, alone or mixed with powdered limestone or with a blend of limestone and sodium dichromate, of powdered chromite ore roast, of a mixture of equal parts of zinc chromate, barium chromate and lead chromate, and of green chromium oxide by mice, rats, guinea pigs and rabbits, failed to result in the development of cancers of the lung in the exposed animals.

Similarly negative was the repeated intratracheal injection into rats of suspensions of zinc chromate, strontium chromate and calcium chromate in gelatin solution.

The intramuscular and intrapleural implantation of chromic chromate, zinc yellow, strontium chromate, calcium chromate, sintered calcium chromate, chromite ore roast residue, lead chromate, barium chromate, and chromium acetate, on the other hand, caused the production of sarcomas and carcinomas at the sites of deposition. The carcinogenic potency of the various chromium compounds tested decreased in the order given from a maximal cancer yield of 75% of the treated rats to a minimal cancer yield of 3%. The relative length of the latent periods of cancers produced by the different compounds was roughly parallel to their potency.

b-1 (cont)

Moderately water soluble hexavalent chromium compounds thus displayed the highest carcinogenic potency.

The demonstration of a cancerigenic response to chromium acetate indicates that not only hexavalent but also trivalent chromium compounds possess carcinogenic properties.

The production of cancers with chromite ore roast residue and with calcium chromate and chromic chromate implies that cancer hazards may be associated not only with the finished products of chromate production for workers employed within a plant but also with intermediate products released as effluents or as discarded residue into the environmental air for the neighborhood population.

The carcinogenic action of chromium compounds is not species specific and not tissue specific, because cancers have been elicited with chromium compounds in rats and mice and from mesodermal and epidermal tissues (connective tissue, muscle tissue, bon e, cartilage, vascular tissue, epithelial lining of bronchi and bronchioli). Whether the epidermal carcinomas found on the snout of about a dozen rats exposed to chromium compounds, especially chromium roast, are attributable to a chromium effect is likely but not established.

Proposed Course: - - This phase of the investigations relating to the production of cancers by chromium compounds has been completed except for the histological examination of a few specimens.

Part B Included: - - (See Part B, NCI-205(b5)

b-2. Chronic Inhalation of a Mixture of Chromite Ore and Limestone Dust on the Respiratory Organs of Mice, Rats, Guinea Pigs, and Rabbits.

Objectives: - - Identical with Serial No. NCI-205(b-1).

Methods Employed: - - The procedure followed in this experiment was described in the 1959 calendar year report.

Major Findings: - - The results are summarized in the following table:

Table 1 - - Death Distribution of Mice, Rats, Guinea Pigs and Rabbits Exposed to Various Chromium Containing Substances by the Respiratory Route.

Agent	Species	No.	Death distribution in months after start							Tumor Yield
			0-6	7-9	10-12	13-15	16-18	19-21	22-24	
Chromite ore roast I	Rat	120	4	1	15	22	23	25	30	5 carcinomas, snout
Chromite ore roast II	Rat	120	1	7	6	15	21	28	42	4 carcinomas, snout

The lungs of the animals exposed to the roasted chromite ore revealed acute and chronic inflammatory changes and black spotting. The histological examination of these organs demonstrated the presence of often large amounts of a dark amorphous pigment in the lumens of the bronchi and alveoli as well as in the interstitial tissue, particularly the subpleural areas (chromitosis). Purulent bronchitis and bronchiectases were often seen in the lungs of the rats. This condition was associated in the lungs with an adenomatous transformation of the peribronchiolar alveoli, which then were lined by a cuboidal or cylindrical epithelium. None of the lungs contained a carcinoma or sarcoma attributable to the material inhaled.

Cancers of the "spontaneous" type, on the other hand, were not infrequently encountered. These were mainly round cell sarcomas of the ileocecal region, reticulum cell sarcomas of the liver, mesotheliomas of the pleura and adenocarcinomas and squamous cell carcinomas of the uterus.

b-2 (cont)
Major Findings (cont)

The only cancers which likely were causally related to the action of chromium were ulcerative lesions about 2 cm. in diameter involving the region of the snout, sometimes including the tissues of the orbit or of the nasal opening. These lesions were found in 5 rats exposed to chromite ore roast #1 and in 4 rats inhaling roast #2. The cancerous process had penetrated in several rats into the maxillary bone and into the oral cavity or had invaded the tissues of the eye.

The cancers were in most cases cornified or noncornified squamous cell carcinomas. Two were sarcomas. Two cancers displayed highly anaplastic structures. These cutaneous cancers of the region of the snout were seen in animals which had been in the experiment for at least 18 months.

Significance: - - Although no lung tumors were produced by the inhalation of the roasted chromite ore which was effective when administered by other routes, these results should not be considered conclusive. Production of cancers in experimental animals has been notably unsuccessful.

Some doubt exists regarding the causal relation between exposure to chromium and the occurrence of the cancers in the region of the snout of rats since similar lesions have been reported by others.

Proposed Course: - - Completed.

PART B Included: - - (See Part B, NCI-205(b5).

b-3. Characterization and Bioassay of Dust from a Chromate Producing Plant.

Objectives: - - When this project was started, a high incidence of cancer of the lung had been observed among workers in chromate producing plants, but the specific agent responsible for this high incidence has not been identified.

Methods Employed: - - Two materials: - pulverized roast matrix and pulverized first-stage residue, were selected for study. The procedures have been described in the 1959 calendar year report.

Major Findings: - - It has been found that the fraction of the residue composed of particles less than 2 microns in diameter has a hexavalent chromium content 3 to 4 times that of the fraction made up of particles greater than 10 microns in diameter.

Fractionation by particle size and analysis of the residue from leaching roasted chromite ore reveal that the smaller particles contain a higher percentage of hexavalent chromium than the larger particles. This increases the acute toxicity of the smaller particle size fractions which reach the lung by inhalation.

The subcutaneous injection in mice of 3, 4-benzpyrene with the residue resulted in a lower incidence of tumors than when the benzpyrene was injected alone or with very insoluble chromic phosphate. The incidence of tumors decreased and the latent period increased with increasing amounts of hexavalent chromium. No promoting effect was observed.

When administered by implantation in sheep fat the residue produced sarcomas in 4 of 70 rats. A fraction of the residue from which most of the water soluble compounds had been removed produced similar tumors in 3 of 52 mice when injected subcutaneously. No tumors occurred in the control groups of the same size.

Evidence that chromium in one or more forms is responsible for the increased incidence of cancer of the lung in chromate workers furnished previously by the production of cancers in rats by several chromium compounds is strengthened further by the occurrence of cancers at the site of implantation in mice receiving two of these compounds.

b-3 (cont)
Major Findings (cont):

calcium chromate and sintered calcium chromate. No tumors were produced by sintered chromium trioxide or sintered calcium chromate when injected subcutaneously in mice using tricaprylin as a vehicle.

By administering calcium chromate in gelatin capsules instead of sheep fat, it has been shown that the latter is not essential to the production of cancers in rats by chromium compounds.

Significance: - - The results of this experiment give additional evidence that the intermediate products in the production of chromium chemicals and the discarded residue may be hazardous.

Proposed Course: - - This project has been completed except for the further observation of a group of rats which in January 1960 received intrapleural implants of 50 mg. of the chromite residue from which most of the water soluble components had been removed. These animals will be observed until they die or for a total period of two years in an effort to obtain more information regarding the hazard of the material that is discarded from the chromate-producing plants.

PART B INCLUDED: - YES

PART B:

Publications: - Payne, Wm. W.: Production of Cancers in Mice and Rats by Chromium Compounds. A.M.A. Archives of Industrial Health, 21:530-535, (June) 1960.

Payne, Wm. W.: The Role of Roasted Chromite Ore in the Production of Cancer. A.M.A. Archives of Environmental Health, 1:20-26, (July) 1960.

b-5. Prolonged Inhalation of Chromic Oxide and of Zinc Chromate, Barium Chromate and Lead Chromate by Rats.

Objectives: - - The chromium compounds listed are some of the most widely used chromium pigments to which an appreciable number of workers have contact by the respiratory route. Handlers of zinc chromate, barium chromate and lead chromate are suspected of having an excessive liability to cancer of the lung. Although three of the four compounds listed are being investigated for carcinogenic properties through their parenteral administration by various routes in experimental animals, the main human contact with them is by inhalation. It appeared, therefore, to be important to study the effects of their inhalation upon the respiratory system of rats.

Methods Employed: - - Sets of fifty rats each are being exposed to the inhalation of chromium oxide (chrome oxide green) and of a mixture of equal parts of zinc chromate (zinc yellow), barium chromate and lead chromate (chrome yellow), respectively. The pigments used consist of a mixture of particles of different sizes, the majority of which is kept below 4 microns in diameter.

Major Findings: - - The results of this experiment are summarized in the following table:

Death Distribution of Mice, Rats, Guinea Pigs and Rabbits Exposed to Various Chromium Containing Substances by the Respiratory Route.

			Death distribution in months after start							Tumor
Agent	Species	No.	0-6	7-9	10-12	13-15	16-18	19-21	22-24	Yield
Yellow Pigments	Rat	72	5	3	9	10	10	17	18	No tumors of lung
Chrome Green	Rat	72	2	3	8	12	11	10	26	No tumors of lung

The lungs of the rats exposed to green chromium oxide exhibited at autopsy, lungs with extensive spotty green deposits. The histologic examination of the lungs of rats inhaling the yellow pigments or chrome green, revealed often peribronchial focal adenomatosis as well as squamous cell metaplasia of the bronchial epithelium lining bronchhectases. None of the lungs of the rats of both series contained a primary cancer. Acute and chronic inflammatory changes in the lungs were often present.

b-5. Prolonged Inhalation of Chromic Oxide and of Zinc Chromate, Barium Chromate and Lead Chromate by Rats
(continued)

Proposed Course: - - Completed.

PART B INCLUDED: - - YES

Serial No. NCI-205 (b-1 through b-5)

Calendar Year 1960

PART B:

Publications: - - Hueper, W. C. and Payne, Wm. W.: Experimental Studies on Chromium Compounds, Their Carcinogenicity and their Importance for Industrial Medicine. Presented before the 13th International Congress on Occupational Health, New York, N. Y., July 1960. To be published in the Proceedings.

Serial No. NCI-206 (a1, a2, d2, d3, d5, d6, d7, d8, d9, d10, & e)

1. OADR
2. Environmental Cancer
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: - Carcinogenic Studies on Food Additives & Contaminants

a-1. Carcinogenic Studies on coffee soots. Polycyclic aromatic hydrocarbons in coffee soots. *

a-2. Carcinogenic Studies on coffee soots. Bioassays of coffee soots and acetone extracts of coffee soots. *

*Terminated, but note Part B Included for 1960 calendar year.

d. Carcinogenic Studies on Aromatic Amines and Food Dyes.

2. Biochemical Investigations on the Metabolism of the Food Dye, Yellow AB (Bioassay of Yellow AB and four related compounds.

Principal Investigator - - W. D. Conway
Other Investigators - - - E. J. Lethco
W. C. Hueper

3. Aromatic Amine Impurities in Yellow AB and Yellow OB Food Dyes.

Principal Investigator - - W. D. Conway
Other Investigator - - - - E. J. Lethco

(See continuation of studies under "d" succeeding pages)

Man Years (Calendar year 1960)
Total:---------- 6.1
Professional:---- - 2.5
Other:---------- 3.6

d. (cont) Carcinogenic Studies on Aromatic Amines and Food Dyes

4. Metabolic Studies on the Food Dye Light Green SF (FD&C Green No. 2).

Principal Investigator - W. D. Conway
Other Investigator - R. W. Feldstein

(d-4 terminated. No publications.)

5. Metabolic Studies on the Dye, Citrus Red No. 2.

Principal Investigator - W. D. Conway
Other Investigator - E. J. Lethco

6. Metabolic Studies on the Dye: - Pyridium.

Principal Investigator - W. D. Conway
Other Investigator - - R. W. Feldstein

7. Bioassay of Azo Dyes and Their Metabolites by Bladder Implantation.

Principal Investigator - W. D. Conway
Other Investigators - - W. C. Hueper
R. W. Feldstein
E. J. Lethco

8. A Study of the Carcinogenic Properties of the Emulsifier, Polyoxyethylene(8) Stearate.

Principal Investigator - W. C. Hueper
Other Investigator - - Wm. W. Payne

9. Carcinogenic Studies on Arsenic

Principal Investigator - W. C. Hueper
Other Investigator - - Wm. W. Payne

d. (cont)

10. Carcinogenic Studies on 2-Phenylnaphth[1, 2]triazole

Principal Investigator - - W. D. Conway
Other Investigators: - - - W. C. Hueper
R. W. Feldstein

e. Study of Fish Food as a Causative Factor of Hepatomas in Rainbow Trout

Principal Investigator - - Wm. W. Payne
Other Investigator - - - W. C. Hueper

Cooperating Units - - - U. S. Fish & Wildlife Service

a-1. Polycyclic aromatic hydrocarbons in coffee soots.

Terminated. Note Part B Included (publication).

a-2. Bioassays of Coffee Soots and Acetone Extracts of Coffee Soots.

Terminated. Note Part B Included (publication).

d-2. Biochemical investigations on the metabolism of the food dye, Yellow AB(Bioassay of Yellow AB and four related compounds).

Objectives: - - When carrying out a study to determine whether or not long-term-exposure to a substance is harmful, it is well to consider not only the purified substance itself, but also:-1) impurities present in the commercial material; 2) derived compounds which may be produced when the substance comes into contact with other chemical agents before ingestion into an organism and 3) derived compounds, or metabolites produced after the original substance is ingested. Consequently in our investigation of the hazards which might be associated with the use of the dye Yellow AB in food, four simple derivatives as well as a purified sample of the dye itself were tested for carcinogenic activity by subcutaneous injection into mice. The chemical names of the compounds along with abbreviated terminology and a note on their mode of formation are given in the following table:

d-2 (cont)

Compound	Abbreviation	Mode of Formation
Yellow AB(1-phenylazo-2-naphthylamine)	YA	
2-Phenylnaphth[1, 2]triazole	PN	Oxidation of YA
1-Anilino-2-methylnaphth[1, 2]imidazole	AM	Coupling of YA with acetaldehyde
1, 2-Diaminonaphthalene	DA	Reductive cleavage of YA
2-Methylnaphth[1, 2]imidazole	MN	Coupling of DA with acetic acid

Methods Employed: - - The compounds, as solutions or suspensions in glycerine or purified tricaprylin were administered subcutaneously above the right hip to C-57 mice for a period of 84 weeks. Animals were examined at the time of death for a period of 105 weeks. Animals were divided into three series: - controls, a low-dose-series (series 1) and a high-dose-series (series 1A). During the course of the experiment it was necessary to modify the dosage schedule as indicated in the following table:

Series	Total Animals	Dosage Schedule	Total dose in 84 weeks
1	40	0. 1 mgm. per week for 46 weeks 0. 2 mgm. every 2 weeks thereafter	8. 2 mgm. in 64 injections
1A*	20	0. 1 mgm. per week for 26 weeks 4. 0 mgm. at intervals of 4 to 8 weeks thereafter	66. 6 mgm. in 42 injections
Controls	60	0. 1 ml. of vehicle following same schedule as series 1	

* Administration of Compound DA in this series was discontinued after 40 weeks. A total of 26. 6 mgm. was given in 32 injections.

In all cases the dose was administered in 0. 1 ml. of vehicle. Glycerine was used as the vehicle for the first 13 injections at which time it was noted that this material produced hematuria. Purified tricaprylin was used for injection 14 and thereafter. It was necessary to purify the tricaprylin because the commercial material (Eastman Kodak) contained an impurity (probably capric acid) which reacted with 1, 2-diaminonaphthalene and Yellow AB, two of the compounds being tested.

Major Findings: - - Tumors detected at autopsy are listed in the following table:

d-2 (cont)

Compound	Animals with Tumors	Series	Time of Death (weeks)	Site of Tumor	Description of Tumor
Vehicle	2	Control	44	injection site	.75 cm. diam., medullary
			68	injection site	1 cm diam., soft, mushy, partly medullary
AM	3	1	41	injection site	1.5 cm diam., white, medullary, invasive in surrounding tissue
		1	44	injection site	1.5 cm diam., medullary
		1	78	rt. shoulder	1.5 cm diam., medullary
MN	2	1	32	injection site	1 cm. diam., yellow, medullary
		1A	81	liver	1 cm diam., white, medullary
YA	2	1	43	ovary	1.5 cm diam. red cystic tumor
		1A	65	ovary	1 cm. diam. firm red tumor
PN	1	1	81	injection site	2.5 cm diam.

Injection site tumors which were produced in tests of three of the compounds may be due to the vehicle since two injection site tumors were noted in the control series. In the series receiving compound MN, one liver tumor was observed and in the series receiving YA, two ovarian tumors were observed.

An interesting observation, which may have practical significance, has been made in the course of this experiment. The death rate for animals receiving compound AM appears to be significantly lower than the death rate for control animals. Administration of compound AM was associated with an increase of the lifespan of the animals by 8 to 11 weeks. This increased lifespan seems to be due to a lowering of the incidence of liver abscesses in the animals. At death, 15% of the control animals had liver abscesses while only 5% to 10% of the animals receiving AM had this infection. These facts are summarized below:

d--2(cont)

Compound	Series	Average Lifespan of Animals (weeks)	Incidence of Liver Abscesses at Autopsy
Vehicle	Control	57.1	15%
AM	1	64.9	5%
AM	1A	68.4	10%

Significance: - - At the present time, Yellow AB is not allowed for use in food or cosmetics. If abscesses are the primary cause of death in those animals afflicted with such lesions at death, the lower death rate in the group of animals receiving compound AM may result from prevention of these abscesses. The possible chemotherapeutic usefulness of the compound deserves future consideration.

Proposed Course: - - Since Yellow AB has been removed from the list of dyes certified for use in food, further work on this problem is not contemplated at the present time.

PART B INCLUDED ---NO

d-3. Aromatic Amine Impurities in Yellow AB and Yellow OB Food Dyes

Principal Investigator - - W. D. Conway
Other Investigator - - - E. J. Lethco

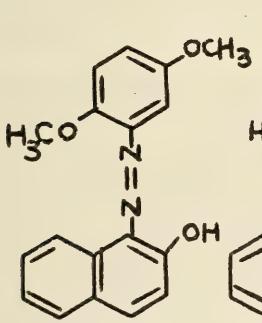
This investigation was completely described in last year's report. Part B was not available at that time but is now included.

PART B INCLUDED - - - YES

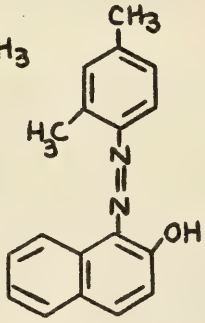
d-5. Metabolic Studies on the Dye, Citrus Red No. 2

Objectives: - - Citrus Red No. 2 is an azo dye having the structure shown below. It has been certified as a substitute for FD&C red No. 32 for use in dyeing Florida oranges. The latter dye has been banned because of its high toxicity.

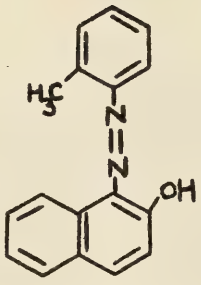
Two structurally similar dyes, Orange SS and 1-benzeneazo-2-naphthol have been shown by Jull and co-workers to induce tumors when implanted in the bladders of mice. All four dyes on reductive cleavage will produce 1-amino-2-naphthol which has also been shown to be carcinogenic to bladder epithelium. An isomeric aminonaphthol, namely, 2-amino-1-naphthol has long been recognized as a carcinogenic metabolite of beta-naphthylamine.



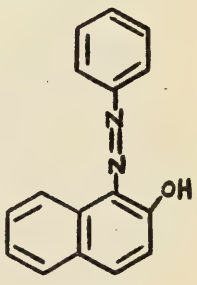
Citrus Red No. 2



Formerly FD&C Red No. 32



Orange SS Formerly FD&C Orange No. 2



1-Benzeneazo-2-naphthol

It is the object of this investigation to study the metabolism of citrus red no. 2. Metabolites formed, particularly ortho-aminonaphthols, will be identified and tested for carcinogenic activity.

Methods Employed: - - Commercial Citrus Red No. 2 (hereafter referred to as citrus red) was obtained from American Cyanamid Co. Dye labeled with radioactive carbon-14(1-[2,5-dimethoxy-phenylazo]-2-naphthol-8-C^{14}, hereafter referred to as citrus red-C^{14}) was synthesized from commercially available 2-naphthol-8-C^{14}. The synthetic material had a specific activity of approximately 16 microcuries/

d-5 (cont)

gram.

Purina Rat Chow containing up to 2% dye was administered to NIH black rats. Special food containers were used which minimized the loss of food. Urine and feces were collected separately in containers maintained at a temperature of 0°C. to prevent decomposition.

Unchanged citrus red was recovered from the feces by extraction with alcohol and determined spectrophotometrically. Colored urinary metabolites were determined spectrophotometrically. Colorless urinary metabolites were determined by radioactivity analysis.

Urine was fractionated by solvent extraction and paper chromatography. Fractions were examined by spectrophotometric and chemical methods.

Major Findings: - - The urine of animals fed commercial citrus red is red in color. Spectrophotometric analysis indicates that the colored materials excreted represent less than 1% of the dye consumed. Fractionation by paper chromatography indicates the presence of at least 7 colored materials although one of these accounts for over half of the total color. It is possible that some of these colored materials are due to impurities in the citrus red or to decomposition of other colored metabolites. Commercial citrus red was shown by chromatography to contain several colored impurities totaling about 2% of the commercial dye.

Radioassay of urines from animals fed citrus red-C^{14} shows that the colored metabolites excreted represent only a small fraction of the total urinary metabolites. A summary of data obtained using radioactive dye is given in the following table:

Dose (mgm)	Metabolites Excreted by Rats in 3-Day Period: Unchanged Dye Recovered from feces (mgm)	Dye accounted for in urine: Colored Metabolites (mgm)	Dye accounted for in urine: Colorless Metabolites (mgm)
99	71	0.3	9
50	33	0.1	7
25	8	0.4	8
10	0	0.05	5

d-5 (cont)

The percentage of the dose which appears as urinary metabolites increases as the dose is decreased. Nine percent of a 99 mgm. dose is excreted by this route.

1-Acetamido-2-naphthol was identified in an ether extract of urine which had been treated with B-glucuronidase.

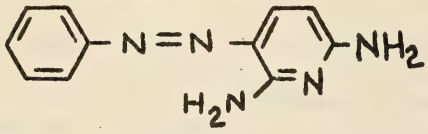
Significance: - - Dyes which are very similar chemically to citrus red have been reported to be carcinogenic. A simple reduction product of these dyes and of citrus red itself has also been shown to have carcinogenic properties. The carcinogenic hazard which might arise through addition of citrus red to the human diet has not been thoroughly investigated. Results obtained in this investigation will help to establish whether such a hazard exists. Results already obtained show that a large fraction of the dose of citrus red is excreted in the urine. This fraction is larger after ingestion of very small amounts of dye. This fact points out the necessity of studying the metabolism of food dyes at very low dose levels, comparable to those levels at which the dyes are present in human food.

Proposed Course: - - This investigation will be continued. Urinary metabolites will be identified and assayed for carcinogenic activity. Citrus red itself is being tested for carcinogenic activity using the bladder implantation technique described elsewhere in this report.

PART B INCLUDED - - - - NO

d-6. Metabolic Studies on the Dye Pyridium

Objectives: - - Pyridium is an azo dye having the following structure:



The dye has analgesic and antiseptic properties and has been used for many years, either as the hydrochloride or in combination with sulfonamides and other antibiotics for treatment of urinary infections.

Boyland and co-workers (Brit. J. Cancer. 212 (1957)) have shown that pyridium will induce bladder tumors when cholesterol pellets containing the material are introduced into the bladders of mice. This finding casts some doubt on the wisdom of using this material for the treatment of chronic urinary infections.

A search of the literature did not reveal any metabolic studies on pyridium.

It is the object of this investigation to determine the nature and quantity of urinary metabolites produced following oral administration of the dye to animals and man. These metabolites will then be assayed for carcinogenic activity.

Methods Employed: - - Pyridium hydrochloride was administered to rats either by capsule or in aqueous solution via stomach tube. Urine and feces were collected separately. Colored material in the urine was determined spectrophotometrically and expressed as unchanged pyridium. Urine was also examined by paper chromatography. Feces were extracted with water and butanol to recover unchanged pyridium which was measured spectrophotometrically. An attempt was also made to determine whether or not there was an increase in the ethereal sulfate and glucuronic acid content of urine following administration of the dye.

Major Findings: - - Spectrophotometric analysis of the urine of two NIH black rats following administration of 16 mgm. of pyridium hydrochloride indicates that within 3 days 20% of the dose is excreted as a colored conjugate. No free pyridium could be extracted

d-6 (cont)

from the urine, nor was any found on paper chromatography of the urine. No more than 8% of the dose could be found in the feces. No further excretion of colored material occurs after the second day.

On paper chromatography of concentrates obtained from the urine of dosed animals, a spot corresponding to a salt of p-aminophenylsulfuric acid can be detected in several solvent systems. Since this substance is a metabolite of aniline, the dye may be split in the body forming aniline and a triaminopyridine.

Attempts to detect an increase in ethereal sulfate and glucuronic acid following administration of the dye to four rats were inconclusive. There appears to be a small increase in ethereal sulfate excretion corresponding to 5 or 10% of the pyridium hydrochloride administered but the variation in the daily ethereal sulfate content of normal rat urine was so great that an accurate estimation of the pyridium-sulfuric acid conjugates could not be made. The variation in normal glucuronide content of rat urine was too extensive for even a rough estimate of the pyridium-glucuronic acid conjugates to be made. Four species of rats and four diets were investigated in an attempt to obtain lower and more constant normal ethereal sulfate and normal glucuronic acid levels but to date these have been unsuccessful.

Significance: - - If pyridium or any metabolite arising from it is carcinogenic, the justification of its further use in the treatment of urinary infections should be questioned. The results of this investigation will assist in determining whether or not a hazard exists when the compound is used medicinally.

Proposed Course: - - A large fraction of the administered dose cannot be accounted for as colored material in the urine and feces. It, therefore, appears likely that pyridium is extensively split in the body. The search for these fragments is greatly simplified by using carbon-14 as a tracer. This investigation will be continued using suitably tagged pyridium. Accurate determinations of the colored and colorless metabolites in the urine and feces will then be made. Metabolites of the dye will be isolated and identified and assayed for carcinogenic activity.

PART B INCLUDED - - - NO

d-7. Bioassay of Azo Dyes and Their Metabolites by Bladder Implantation

Objectives: - - A method for testing materials for carcinogenic activity by surgical implantation of pellets containing the material into the bladders of mice was developed by Jull (1951) and has been used with notable success since then by Jull, Bonser, Boyland and others.

These workers have used pellets prepared from either paraffin wax or cholesterol. Both of these substances produce some tumors in control animals, thereby greatly reducing the sensitivity of the method. It is not certain whether these materials are carcinogenic in themselves or whether they contain a carcinogenic impurity. Both substances are difficult to purify.

It is the object of this investigation to test azo dyes and their metabolites by the bladder implantation technique using pellets prepared from a synthetic wax and mixtures of the wax with powdered cellulose. By mixing varying amounts of powdered cellulose with the wax a pellet is produced which is more permeable to water. The latter type of pellet may be more suitable for assaying compounds having very low water solubility.

Methods Employed: - - Hexadecyl palmitate (melting point 52°C.) was prepared by reaction of hexadecanol with palmitoyl chloride followed by recrystallization from alcohol. Pellets were prepared by compression of mixtures of the powdered compounds with the vehicle using a stainless steel die. Pellets were tablet-shaped, 3.2 mm. (1/8 inch) in diameter and 1.4 mm. thick, and weighed approximately 10 mgm.

In order to determine the amount of cellulose which should be incorporated into the pellet to obtain a water permeability approximating that of cholesterol, several pellets were prepared containing 20% 3-hydroxyanthranilic acid (3HAA) and varying amounts of hexadecyl palmitate (HP) and Whatman cellulose powder for chromatography, standard grade (Cel). These were incubated at 38°C. with 1 cc of water. The rate of extraction of 3HAA from the pellet was determined spectrophotometrically and compared with published data for pellets prepared from 3HAA plus cholesterol as illustrated in the following table:

d-7 (cont)

Release of 3-Hydroxyanthranilic acid (3HAA) from Cholesterol Pellets and from Pellets Composed of Hexadecyl Palmitate (HP) Plus Varying Amounts of Cellulose (Cel).

Pellet Composition %			% of 3HAA Extracted after		
3HAA	Cholesterol *		10 days	30 days	60 days
20	80		32	60	75
3HAA	HP	Cel			
20	78	2	5	6	7
20	71	9	6	9	11
20	55	25	14	22	30
20	35	45	20	34	45
20	30	50	35	68	87

*Allen et al., Brit. J. Cancer. 212 (1957)

This data illustrates that the rate of release of compounds by the pellet into the urine, and hence the degree of exposure of the bladder wall to the compound, may be controlled over a wide range by incorporation of powdered cellulose into the pellet.

Several compounds are being tested by bladder implantation using pellets composed of hexadecyl palmitate and hexadecyl palmitate plus cellulose. Sixty (60) female C-57 mice were used in each group except the controls where 90 mice were used. The compounds and composition of the pellets are shown in the following table:

Compounds Being Tested by Bladder Implantation

Chemical Name	Common Name	Pellet Composition (%) Compound	Hexadecyl Palmitate	Cellulose
1-(2-methylbenzeneazo)-2-naphthol	Orange SS	15	85	--
		15	35	50
1-(2,4-dimethylbenzeneazo)-2-naphthol	Formerly FD&C Red No. 32	15	85	--
		15	35	50
1-(2,5-dimethoxybenzeneazo)-2-naphthol	Citrus Red No. 2	15	85	--
		15	35	50
1-anilino-2-methylnaphth[1,2]imidazole	----	15	85	--
2-methylnaphth[1,2]imidazole	----	15	85	--
Controls	----	--	100	--
		--	50	50

d-7 (cont)

Major Findings: - - It is too soon to evaluate the results of this experiment

Significance: - - The use of hexadecyl palmitate and mixtures of hexadecyl palmitate with cellulose may increase the sensitivity of this method for evaluating carcinogenicity by reducing or eliminating the incidence of bladder tumors in the control animals. The results obtained by testing various dyes and their metabolites will be helpful in determining whether or not these dyes are innocuous to humans.

Proposed Course: - - If after further study this technique continues to appear useful, numerous other compounds will be tested. In particular, ortho-aminonaphthols formed on reductive cleavage of several FD&C azo dyes will be tested.

PART B INCLUDED - - - NO

d-8. A Study of the C arcinogenic Properties of the Emulsifier, Polyoxyethylene(8)Stearate.

Objectives: - - The Food Protection Committee of the National Research Council concluded in 1953(National Academy of Sciences-National Research Council Publication 280) that "available data fail to demonstrate that polyoxyethylene stearates are safe for use in foods under all patterns of dietary consumption and for all segments of the population".

Subsequently additional data were reviewed by the Subcommittee on Toxicology, which reported (NAS-NRC Publication 646), "Every animal (rat) that had a bladder tumor also had at least one bladder stone. It seems likely that the tumors were caused by chronic irritation of the bladder epithelium by the stones". The Subcommittee concluded, "the use of polyoxyethylene(8)stearate at levels not greater than 0.05 percent in the human diet would be safe". It does consider the chemical to be a carcinogen.

This project was undertaken to determine whether the material is carcinogenic to animals and whether bladder tumors without bladder stones could be produced by the administration of the compound. To investigate the possibility that the compound is a promoting agent the

d-8 (cont)

experimental plan included the injection of the compound with a known carcinogen.

Methods Employed: - - POE(8)S is being administered to animals as follows:

1. Feeding	60 rats	25%	POE(8)S in food
	60 rats	10%	ditto
	60 mice	25%	ditto
	60 mice	10%	ditto
	60 mice	5%	ditto
2. Intramuscular Injection	30 rats	100 mg.	in thigh
	30 mice	25 mg.	in thigh
3. Intrapleural Injection	30 rats	50 mg.	(repeated monthly)
4. Intra-pouch Administration	30 hamsters	400 mg.	in gelatin capsule
5. Intravesical administration	50 rats	20 mg.	in bladder
6. Subcutaneous Injection in nape of neck with 3,4-benzpyrene	60 mice	0.025 mg.	benzpyrene and 25 mg. POE(8)S
	60 mice	0.05 mg.	benzpyrene and 25 mg. POE(8)S
	60 mice	NO	benzpyrene and 25 mg. POE(8)S

Significance: - - This experiment is a part of this Section's study of carcinogenic effects of food additives. Because polyoxyethylene(8)stearate may be used as an emulsifier in foods that are consumed daily throughout a person's lifetime - bread, rolls and frozen dairy products - it is important that there should be no doubt regarding the safety of compound when ingested.

d-8 (cont)

Major Findings: - - Among the 50 rats which were given intravesical administrations of POE(8)S, at the end of one year bladder stones had been found in three while there were 36 survivors. Mortality has been low among all groups of animals.

Proposed Course: - - The feeding will be continued for a total period of two years. All animals will be observed for a minimal period of two years after the initial administration of the material.

PART B INCLUDED: - - NO

d-9. Carcinogenic Studies on Arsenic.

Objectives: - - Although repeated efforts to produce cancers in experimental animals have been unsuccessful there is considerable epidemiologic and clinical evidence which indicates that arsenic is carcinogenic for man. Recently it has been observed in Europe that vineyard workers who used arsenical pesticide sprays and who consumed large volumes of wine contaminated by sprays containing arsenic, experienced a high incidence of cancer of several sites (skin, lung, liver). Since arsenic may be ingested not only with contaminated foods but also in water polluted by industrial wastes containing arsenic, its presence in man's diet is of increasing concern.

The observation on the vineyard workers who consumed arsenicals in an alcoholic solution suggested that the introduction of the chemical in this form might finally result in a successful production of experimental arsenic cancers. For this reason the experimental conditions used simulate those experienced by the workers. The following experimental scheme was devised:

Methods Employed: - - Arsenic in the form of As_2O_3 is administered in drinking water to 200 - C57 Black mice and 200 Bethesda Black rats. Both the rats and mice are divided into four groups of 50 each so that one group of rats and one of mice receive one of the following solutions:

d-9 (cont)

1. Tap water containing As_2O_3
2. 12% aqueous solution of ethyl alcohol containing As_2O_3
3. 12% aqueous solution of ethyl alcohol
4. Tap water

The dose of As_2O_3 is being gradually increased but is kept to a level that produces no acute toxicity.

Animals are weighed regularly and are observed for the presence of tumors.

Major Findings: - - All mice kept on arsenic-containing drinking fluids have died without showing signs of cancerous reactions or of chronic arsenicism. The rats withstood well the progressive increase of arsenic trioxide in the drinking fluid. About 50% of their original number are alive after being about 18 months in the experiment. Cancers were seen in rats of all four series. They were of the sites commonly seen in this strain and were not more frequent among the rats belonging to the arsenic groups than in those given plain water or a 12% alcohol solution.

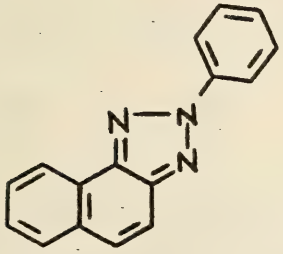
Significance: - - The understanding of the role of arsenic in carcinogenesis as well as its role as a food contaminant would be enhanced greatly if a method could be found to produce cancers in experimental animals with arsenic.

Proposed Course: - - The exposure will continue for a minimal period of one year if the animals survive.

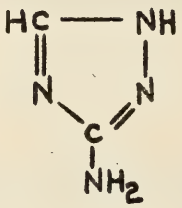
PART B INCLUDED: - - NO

d-10. Carcinogenic Studies on 2-Phenylnaphth[1, 2]triazole

Objectives: - - A herbicide (3-amino-1,2, 4-triazole) used for treatment of cranberry bogs has recently been shown to cause thyroid tumors in rats when fed at a level of 15 ppm. We have tested a similar compound, 2-phenylnaphth[1, 2]triazole by subcutaneous injection in mice with negative results. Structures of these materials and abbreviated terminology are shown below:



2-Phenylnaphth[1, 2]triazole
Abbreviation:PNT



3-amino-1, 2, 4-triazole
Abbreviation: AT

The effect of AT on the thyroid led us to wonder whether PNT might produce a similar result.

Methods Employed: - PNT was prepared by oxidation of 1-phenylazo-2-naphthylamine (Yellow AB) with cupric acetate. The crude material was chromatographed on alumina and recrystallized from aqueous ethanol.

The test diet containing 100 ppm of PNT was prepared by thoroughly mixing an ether solution of the compound with ground Purina Rat Chow and allowing the ether to evaporate.

Ten (10) male and 10 female NIH Black rats are eating this diet continuously.

Major Findings: - - The animals have been tolerating this diet well for 6 months. It is still too early to obtain results.

d-10 (cont)

Significance: - - PNT is produced by simple oxidation of the dye Yellow AB. Although Yellow AB is no longer allowed as a food or cosmetic dye in this country, it is still permitted in some foreign countries. It was widely used here for coloring oleomargarine and would be subject to oxidation during the cooking, especially frying, of foods containing the margarine. Since in our previous investigation of this compound we used subcutaneous administration and since the thyroid was not extensively examined, it was deemed worthwhile to conduct this investigation.

Proposed Course: - - The test diet will be administered until the death of all animals.

PART B INCLUDED - - - - NO

e. Study of Fish Food as a Causative Factor of Hepatomas in Rainbow Trout

Objectives: - - Early in 1960 rainbow trout reared in hatcheries in the Western United States were observed to have an unusually high incidence of tumors of the liver. The first observations were made on yearling fish, but the ailment was later found to be present in older fish in the Eastern States as well as in the West. Prevalences as high as 70 to 80% have been observed in some groups of fish. Observations made by local fishery and health department workers indicated that diet is involved. During the last few years a dry diet has come into wide use in Federal, State and private hatcheries. Some factor or component of this dry diet is suspected as the causative agent, but other possibilities exist, such as virus and species susceptibility.

Control of this ailment in the fish cannot be undertaken effectively until the causative factor is isolated. In the meantime, private feed producers and trout growers are facing a serious economic problem while the Federal and State fisheries authorities are concerned about their propogation and stocking activities and possible health implications.

Methods Employed: - - The activities of the Section have taken two approaches - - assistance to other groups and direct investigations.

e. (cont)
Methods Employed (cont)

Assistance has been rendered by Dr. Hueper and others at NCI in the planning of research programs to be carried on by agencies who have extensive facilities for rearing fish. Other activities include the collection of information and determination of the extent of the problem.

Limited laboratory research has been undertaken using mice, rats, and a few trout. Two commercial fish diets are being fed to mice and rats to determine whether the liver tumors can be produced in these animals by diets that are suspected of causing the lesions observed in trout. An extract prepared from one of the diets is being administered to mice and will be given to fish, as soon as additional ones are available.

One of the antioxidants used widely in fish foods is being administered to mice by feeding and injection and to trout by incorporating it into the beef liver diet. Trout serving as controls receive beef liver only.

Major Findings: - -Information that has been collected indicates that not only dry commercial diets are involved but also diets that contain dry components (fish meal, cottonseed meal, wheat middlings, etc.) as well as meat. The tumors have been found in rainbow trout in all parts of the United States where they are raised and a few years ago in Italy. Preliminary data indicate that the tumors are occurring in rainbow trout only and seldom in brook or brown. Hence species susceptibility may be of importance.

Results of laboratory experiments are not yet available.

Significance: - - This problem affords not only an opportunity to study a fish disease of unusual proportions, but also is creating unusual interest in environmental cancer. The investigations related to this problem may reveal new avenues of approach to other environmental cancer problems, particularly those relating to diet, especially since fish oils are extensively used in human foods.

Proposed Course: - - The laboratory animal work will be continued until the animals have been observed for two years. Additional trout will be obtained as soon as they are available from the hatchery.

PART B INCLUDED - - - NO

PART B:

Publications:

a-1.

Kuratsune, M. and Hueper, W. C.: Polycyclic Aromatic Hydrocarbons in Roasted Coffee. J. N. C. I. 24:463-469, 1960.

a-2.

Hueper, W. C. and Payne, Wm. W.: Carcinogenic Studies on Soot of Coffee-Roasting Plants. Arch. Pathol. 69:716-727, 1960.

d-3.

Conway, W. D. and Lethco, E. J., Aromatic Amine Impurities in Yellow AB and Yellow OB Food Dyes. Anal. Chem. 32: 838-841, 1960.

Serial No. NCI-209
1. OADR
2. Environmental Cancer
3. Bethesda, Md.

Calendar Year 1960

W. C. Hueper, M. D.

Medicolegal, Consultative, Advisory and Educational Activities in Environmental Cancer

The rapid growth in factual knowledge on the actual and potential exogenous causes of human cancers has brought about an augmented awareness of environmental cancer hazards to all specially interested parties, such as members of the medical profession, industrial management, labor organizations, public health agencies, members of the legal profession, birth control organizations, agricultural and food processing agencies, as well as to the general public. Environmental cancer hazards and cancers have become a recognized public health problem of distinct stature. The many and increasingly frequent inquiries on this subject made by intragovernmental and extragovernmental, public and private parties to the Environmental Cancer Section require a constant collection of pertinent data from the literature and their competent interpretation in the light of the old accumulated and newly acquired knowledge.

Man Years (calendar year 1960)
Total: ------- 0.7
Professional: - 0.2
Other: -------0.5

Medicolegal Activities:

Consultative cooperation with the Bureau of Employees' Compensation, U. S. Department of Labor, was continued with the exception of a brief period during which I withdrew from these activities because of temporary censorship applied to two medicolegal papers prepared by me for publication.

Assistance has been given on several occasions to members of the Food and Drug Administration on problems of cancer hazards from food additives and contaminants and drugs. They were supplied with reprints dealing with such matters, including copies of the still unpublished paper,

"Consumer Goods and Cancer Hazards", since it gives the most complete and competent review of this important problem, including an extensive bibliography.

Medicolegal advice was given to a number of attorneys in connection with litigations on traumatic and occupational cancer claims. Likewise, free expert testimony was offered upon request to several industrial concerns on questions of occupational cancer hazards and compensation suits, and to several private parties. Members of Congress were supplied on request with literature pertaining to questions of cancer hazards from food additives and contaminants and of a preventive control of environmental cancers.

Significance: - - The activities outlined are essentially of an educative and informative nature. It is hoped that they may aid in alerting an increasing number of individuals to the serious threat to public health created by some products of modern industry.

Prospective Course: - The past activities in this field will be continued whenever they are requested or needed.

Publications:

Listing of 1960 publications on medicolegal and sociologic aspects:

Hueper, W. C., Carcinogens in the Human Environment: Arch. Pathol., in press.

Hueper, W. C., Medicolegal Considerations of Occupational Cancers. Lawyers' Medical Cyclopedia, in press.

Hueper, W. C., Medicolegal Considerations of Non-Occupational Cancers, Lawyers' Medical Cyclopedia, in press.

Hueper, W. C., Der Berufskrebs. In E. Baader, Handbuch der gesamten Arbeitsmedizin, Urban & Schwarzenberg, Munich, vol. 2, in press.

Hueper, W. C., Carcinogens in the Human Environment. Presented Science Writers' Seminar, Louisville, Ky., sponsored by the American Cancer Society, March 1960. To be published in the Proceedings.

Publications (cont)

Hueper, W. C., Occupational Cancers with Special Reference to Occupational Cancer Hazards to Laboratory Personnel. Presented annual meeting Medical Technologists, Atlantic City, N. J., June 1960. To be published in American Journal of Medical Technology.

Hueper, W. C., Medicolegal Considerations of Occupational Cancers, presented 14th Annual Convention National Association for Claims and Compensation Attorneys, San Francisco, July 1960. To be published in the Proceedings.

Hueper, W. C., Cancer Hazards from Natural and Artificial Water Pollutants. Presented Conference on Physiological Aspects of Water Quality, Washington, D. C. September 1960. To be published in the Proceedings.

Hueper, W. C., Potential Cancer Hazards from Cosmetics to Producers and Consumers. Presented New York City, Annual Meeting of N. Y. Chapter of Society of Cosmetic Chemists, September 1960. To be published in the J. Society of Cosmetic Chemists.

Hueper, W. C., Paranasal Sinus Cancers. Presented Tokyo, Japan, October 1960, before the Symposium on Geographic Pathology, International Union Against Cancer. To be published in ACTA.

Honors: - Advisory Editor, Cancer- Progress Volume, 1960, Editor - R. W. Raven, London, England, Butterworth and Co., Ltd., 242 pp.

Speaking Engagements: -

1. Science Writers' Seminar sponsored by the American Cancer Society, Louisville, Ky. March 1960. Invitation by Dr. H. S. Diehl, Vice President for Research and Medical Affairs. Subject:- "Carcinogens in the Human Environment".

2. By invitation of Dr. E. Phillips, Vice Pres. American Association of University Women, Washington, D.C. March 1960, presented talk before the Kensington Branch. Topic: "Consumer Goods and Potential Cancer Hazards".

Speaking Engagements (cont)

3. By invitation of P. Black, Secy. 20-30 Club, All Souls Church, Washington, D. C. February 1960, spoke on "Cancer Producing Agents in the Human Environment".

4. By invitation of Dr. N. E. Needle, Medical & Chirurgical Faculty State of Maryland, Baltimore, conducted Round Table Discussion on "Cancer Hazards in Industry", April 1960.

5. Spoke before the Wayfarers' Club, Unitarian Church, by invitation of program director, Mr. H. Allion on the subject of "Potential Cancer Hazards". Washington, D. C., April 1960.

6. Attended meeting of NAS-NRC, Subcommittee on Atmospheric and Industrial Hygiene of the Committee on Sanit. Eng. & Environment, Washington, D. C. as liaison representative of USPHS, May 1960.

7. Attended Research Session at Air Pollution Control Association Annual Meeting, Cincinnati, May 1960 , at the invitation of Dr. John R. Goldsmith, California Department of Public Health, Session Arranger for Research Session . Presented paper entitled: "Carcinogenic Bioassays on Air Pollutants".

8. Attended Industrial Cancer Congress, Atlantic City, June 1960, by invitation of the Chairman, Dr. H. Stewart, The American Society of Medical Technologists. Title of presentation: "Occupational Cancers with Special Reference to Occupational Cancer Hazards to Laboratory Personnel".

9. Attended the Industrial Health Conference , American Industrial Hygiene Association, April 1960, held in Rochester, N. Y. by invitation of Dr. John Zapp, Session Arranger on Toxicology, at which time presented, "Carcinogenic Studies on Water-Insoluble Polymers".

10. By invitation of Dr. Irving R. Tabershaw, Chmn. Scientific Program Committee, 13th International Congress on Occupational Health, New York City, July 1960, presented paper entitled:-"Experimental Studies on Chromium Compounds, Their Carcinogenicity and Their Importance for Industrial Medicine".

Speaking Engagements (cont)

11. By invitation of Mr. B. Marcus of Marcus, McCroskey, Finucan and Libner, Michigan, attended the Nat. Assoc. for Claims and Compensation Attorneys' Convention, San Francisco, July, 1960. Title of presentation: - "Medicolegal Considerations of Occupational Cancers".

12. Attended the Belli Seminar, San Francisco, July 1960, by invitation of Mr. M. Belli of Belli, Ashe and Gerry presenting a paper entitled, "Medicolegal Aspects of Non-Occupational Environmental Cancers".

13. Spoke before the COSTEP Medical, Dental and Veterinary Medicine Students, Clinical Center, August 1960, at the invitation of Mr. J. A. Staton, Chief, Clinical & Professional Education on the topic, "Carcinogenis Agents in the Human Environment".

14. Addressed the New York Chapter of the Society of Cosmetic Chemists, New York City, September 1960 at the invitation of Mr. J. M. Longfellow, Chairman, on the subject of:- "Potential Cancer Hazards from Cosmetics to Producers and Consumers".

15. Participated in the Conference on Physiological Aspects of Water Quality, Washington, D. C. SEPTEMBER 1960 . Title of paper: "Cancer Hazards from Natural and Artificial Water Pollutants".

16. Spoke on "The Role of Occupational Carcinogens in the General Cancer Panorama"; Topic during round table discussion "Causative Correlation between the stomach ulcer and the stomach cancer", Tohoku University, Sendai, Japan, October 1960. Invited by Dean.

17. Attended and participated in meetings of International Union Against Cancer: - Conference on Cancer Control, Study Group of Food Additives and Symposium on Geographic Pathology, Tokyo, Japan, October 1960. Title of presentation last named Symposium "Cancers of the Paranasal Sinuses".

18. Presented address on "Industrial Cancers" before the Institute of Science and Labor, Tokyo, Japan, Oct. 1960.

19. By invitation of the Dean, Kyushu University, Fukuoka, Japan, presented address on "Air Pollutants and Cancer of the Lung, October, 1960.

Speaking Engagements (cont):

20. Participated in 1-hour Canadian Broadcasting Program (radio link between Washington, D. C. and Toronto), Station WRC, Dec. 1960. Title - Food Additives.

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1. OADR
2. Environmental Cancer
3. Bethesda, Md.

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Individual Project Report
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PART A:

Project Title: - Studies on Occupational and Environmental Respiratory Cancer Hazards Caused by the Inhalation of Carbonaceous Air Pollutants.

*a-1. Inhalation of Carbonaceous Air Pollutants. Coke oven tar and petroleum asphalt.

*a-2. Inhalation of Carbonaceous Air Pollutants. Cutting Oils.

*Project terminated in 1959. See Part B - Publications 1960 calendar year.

b. Urban Air Pollutant Study.

Principal Investigator -- W. C. Hueper
Other Investigator --- Wm. W. Payne
Cooperating Investigators - Paul Kotin and H. Falk, University of Southern California; E. C. Tabor, E. Sawicki, R. A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

Man Years (calendar year 1960)
Total: ---------- 2.0
Professional: -- 0.4
Other: ------- 1.6

b. Urban Air Pollutant Study.

Objectives: - -The marked and progressive rise in frequency of lung cancers observed especially among members of urban populations since the turn of the century has become a matter of general concern. It is important that this development has been characterized by marked epidemiologic irregularities concerning the time of its onset, its progression rate, its sex

b. (cont)

distribution, its intensity in different population groups and geographic areas in individual countries as well as in different countries.

The present joint study was undertaken for ascertaining clues concerning some of the chemical agents present in urban atmospheres and possibly responsible for such developments as well as for obtaining information on the reasons underlying the cited epidemiologic regional and demographic irregularities.

Methods Employed: - -Particulate air pollutants were collected on glass fiber filters at a height of not more than 20 feet above street level where possible, in downtown and industrialized areas of the following 8 cities: - New Orleans, Birmingham, Atlanta, Philadelphia, Detroit, Cincinnati, Los Angeles and San Francisco. These particular cities were selected for,

(a) The availability of adequate epidemiologic data on morbidity and mortality rates from lung cancer among their inhabitants.

(b) The existence of, in part, marked differences in these rates among the cities chosen.

(c) The demonstration of considerable variations in the quantities of solvent soluble fractions of air pollutants in different cities.

(d) The demonstration of distinct differences in the chemical composition of the organic fractions of the particulate phase of air pollutants in different cities, depending in part upon specific sources (industries, automobile traffic, coal or petroleum fuel economy).

Since the bioassay work was to be restricted to testing only organic fractions of the particulate phase of air pollutants for carcinogenic potency the following four fractions were selected: - Crude benzol soluble fraction, aromatic hydrocarbon fraction, aliphatic hydrocarbon fraction and oxygenated fraction. The fractions were prepared from composite collections made over one month and, therefore, could be considered to be roughly representative of the atmospheric pollution prevailing in a particular city and at the particular sites during this period. The fractions were sealed into glass ampoules in a nitrogen atmosphere and kept under refrigeration until used.

b. (cont)
Methods Employed (cont)

The fractions were washed from the ampoules with methylene chloride (spectra-analyzed grade). In order to obtain a uniform suspension the proper amount (0.1 ml. for each individual dose of the material) of tricaprylin was added. The methylene chloride was then evaporated under vacuum at room temperature.

The filters used for the collection of the air pollutants were weighed before and after being used for determining the total amount of pollutants collected. They were then extracted with benzol. Through the evaporation of the benzol the "crude benzol soluble fraction" was obtained, the weight of which was ascertained. A portion of this fraction was then used for obtaining the aromatic, aliphatic and oxygenated fractions. Bioassays for carcinogenic potency, according to a predetermined scheme, were performed on the crude benzol soluble fraction and on the aromatic fraction at the National Cancer Institute, while those on the aliphatic and oxygenated fraction were done at the Department of Pathology, University of Southern California.

An attempt was made to administer standardized and equivalent amounts of the various fractions so as to obtain, if possible, comparable results. Taking into account the limited availability of the test materials and possible acute inflammatory and necrotizing effects, it was decided to use 4 mgs. of the benzol extract suspended in 0.1 ml. of tricaprylin (Eastman trioctanoin) as the individual dose to be given once a month into the subcutaneous tissue of the nape of the neck of C57 black mice. Since the amount of aromatic fraction obtained from a particular crude benzol soluble fraction varied with different cities and different batches, it was decided to use that amount of the aromatic fraction which could be extracted from 4 mgs. of the crude benzol soluble fraction at a given time and batch, as the monthly dose. This amount could be calculated, since the total weight of the crude benzol soluble fraction used for the extraction of the aromatic fraction was known for each batch as was the total yield by weight of this fraction.

The single dose of the crude benzol extract as well as its equivalent of the aromatic fraction, were doubled after 11 months. This dosage was maintained until the end of the experiment.

For the testing of each fraction, a total of 72 C57 black mice,

b. (cont) Methods Employed (cont):

about 2 months old, equally divided as to sex, were used. The mice were kept in stainless steel shoebox cages. The cages were examined twice a day for dead animals. Mice which developed tumors at the site of injection were sacrificed when the tumors measured about 1 cm. in diameter. This scheme was adopted since it seemed to be advisable to determine not only the tumor yield for each fraction but also the approximate length of the latent period for judging the relative potency of the various fractions. Since it is difficult to determine the time of the actual beginning of a cancer, it was decided to use for this purpose rather the period at which a given tumor reaches a certain size. The maximal observation period was 2 years. At the end of it the surviving animals were sacrificed.

Two series of mice, one receiving monthly 0.1 ml. of tricaprylin and the second being given 0.2 ml. of this vehicle, served as controls.

In parallel experiments, 50 strain C57 black mice received monthly subcutaneous injections of 0.5 mg. of the oxygenated fraction suspended in 0.2 ml. of tricaprylin which was administered into the nape of the neck. Because the aliphatic fraction was considered to be the least toxic and caustic fraction, 50 C57 black mice received monthly injections of 1 mg. of this fraction suspended in 0.2 ml. of tricaprylin.

Previous investigations of other investigators had demonstrated the presence of 3,4-benzpyrene in the particulate phase of urban air pollutants. The availability of similar information on the air pollutants to be tested in the present study appeared indicated for attempting to determine whether or not a correlation existed between the degree of carcinogenic responses obtained with the various fractions and their relative 3,4-benzpyrene content. Because of the presence of numerous other chemicals in the various fractions, it was possible that the total carcinogenic effect elicited by a fraction not only could depend on its benzpyrene content but also could be influenced in a positive or negative direction by other carcinogenic, cocarcinogenic or anticarcinogenic substances present in the fraction.

For this reason it was necessary to obtain a scale of cancerous responses to graduated doses of 3,4-benzpyrene given as a single dose as well as in 12 monthly fractionated doses injected subcutaneously into the nape of the neck in C57 black mice, using sets of 72 mice for each

b. (cont) Methods Employed (Cont):

dose. Through this scale it was hoped to obtain a method for possibly estimating the approximate share which could be attributed to 3,4-benzpyrene present in a particular fraction in the production of the total cancerous response observed. The scale appeared also to be suitable for expressing the total cancerous response to a particular fraction in terms of standardized doses of 3,4-benzpyrene.

The experimental scheme adopted seemed to permit thus the determination of the absolute and relative carcinogenic potency of various solvent fractions of the particulate phase of air pollutants collected in 8 metropolitan areas in the United States. It was likely that this methodologic approach might also yield some information on the relative share of carcinogenic potency which three subfractions (aromatic, aliphatic and oxygenated) contributed to the carcinogenic potency of the total fraction (crude benzol soluble), and whether or not the sum of the carcinogenic responses of the three subfractions would account for that of the total fraction. Finally, there was a possibility for ascertaining whether the 3,4-benzpyrene content of the crude benzol soluble fraction would account for the total carcinogenic response obtained from all fractions, or whether non-aromatic and non-benzpyrene constituents, or even non-organic chemicals, might contribute a significant share to the carcinogenic potency of air pollutants in different cities.

Major Findings: - - The major findings are recorded in tables 1 - 3.

From the analysis of the data relating to the injection of single and multiple doses of benzpyrene, the following observations were made:

1. For the smaller single doses the number of tumors increased and the the latent period decreased with increased amounts of benzpyrene. As the single dose was increased further a plateau was reached, and an increase in the amount of benzpyrene did not increase the carcinogenic effect.

2. For repeated monthly doses a similar plateau did not occur in the dose range used in this experiment.

3. Except for very small doses, a given total amount of benzpyrene produced a larger percentage of of tumors when it was injected in

Table 1 - - Length of Latent Periods and Cancer Rates in Mice Injected with the Crude Benzol Soluble Fraction and Aromatic Hydrocarbon Fraction of Air Pollutants.

		Latent Period in months	No. of Survivors	No. Cancers	Percentage of Cancers
Atlanta	Crude Benzol	15	41	3	7
	Aromatic	12	55	4	7
Birmingham	Crude Benzol	9	66	5	8
	Aromatic	12	55	2	4
New Orleans	Crude Benzol	12	55	2	4
	Aromatic	12	57	5	9
Los Angeles	Crude Benzol			0	
	Aromatic			0	
San Francisco	Crude Benzol	12	55	3	5
	Aromatic	9	68	1	2
Cincinnati	Crude Benzol	12	55	3	5
	Aromatic	12	58	6	10
Detroit	Crude Benzol	15	35	2	6
	Aromatic			0	
Philadelphia	Crude Benzol	18	41	3	7
	Aromatic	12	62	1	2

Table 2 - 3, 4-Benzpyrene Content of the Crude Benzol Extract of the Particulate Phase of Air Pollutants of Eight American Cities

City	3, 4-Benzpyrene Content Expressed in Micrograms of Grams of Extract												
	Lot 18	Lot 19	Lot 20	Lot 21	Lot 22	Lot 23	Lot 24	Lot 25	Lot 26	Lot 27	Range	Average	Median
Atlanta	340	780	1100	510	700	640	740	690			340-1100	690	700
Birmingham	1100	970	980	1100	1300	1800	1900	1600			970-1900	1300	1200
New Orleans	430	350	340	300	340	500		430			300- 500	380	350
Los Angeles	33	55	49	53	110	100	86	230	150	150	33- 230	100	93
San Francisco	140	190	140	250		240	230	270			140- 270	210	230
Cincinnati	350	960	1200	1200	1500	1900					350-1900	1200	1200
Detroit		1500	1800	880	1300	1100	1700	2000			880-1800	1500	1500
Philadelphia	370	540	820	650	390	470					370- 820	540	510

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Table 3 - - Tumors in Miœ Injected with Varying Amounts of Benzo[a]pyrene in Single and Repeated Monthly Doses. Number of Mice with Tumors at Site of Injection and Number of Survivors after Specified Intervals.

Total Dose mg.		Cumulative No. of Mice with Tumors & No. Survivors								% of Total Animals with Tumors at Site
		3 mo.	6 mo.	9 mo.	12 mo.	15 mo.	18 mo.	21 mo	24 mo.	
Controls	Single	0/69	0/68	0/63	0/39	0/16	0/6	0/5	0/0	0
Controls	Monthly	0/71	0/67	0/46	0/31	0/17	0/2	0/2	0/0	0
0.008	Single	0/71	0/70	0/67	0/54	0/37	0/18	0/9	0/0	0
0.013	Monthly	0/70	0/69	0/68	0/61	0/47	0/13	0/8	0/0	0
0.025	Single	0/71	1/68	6/62	6/50	7/30	7/12	7/7	7/0	10
0.024	Monthly	0/69	0/68	0/66	1/62	1/50	2/34	2/24	2/0	3
0.05	Single	0/72	3/68	5/65	7/47	7/31	7/31	7/5	7/0	10
0.05	Monthly	0/72	0/71	2/69	4/62	4/47	5/33	7/13	7/0	10
0.10	Single	0/71	17/53	17/53	17/48	17/36	17/20	17/15	17/0	24
0.10	Monthly	0/72	0/69	6/61	10/52	12/45	19/31	26/12	27/0	38
0.25	Single	0/70	12/55	18/49	20/46	20/36	20/22	20/13	20/0	28
0.24	Monthly	0/71	4/67	16/52	23/42	32/26	43/9	44/6	44/0	61
0.50	Single	0/71	14/56	18/52	19/43	19/23	19/19	19/12	19/0	26
0.48	Monthly	0/70	2/68	19/51	34/31	46/12	52/4	53/2	53/0	74

36 Male and 36 Female C-57 Black Mice in Each Group

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b. (cont) -Major Findings: (cont)

twelve monthly doses than when it was administered in a single injection. For 0.5 milligrams the effect was almost three-fold.

4. For the repeated injections there was a longer time interval from the first injection to the first appearance of tumors than for the single injections.

These findings show that, at least for mice, a given amount of carcinogen is more effective when it is administered in small increments over a long period of time than when it is administered in a single dose. Conversely, it appears that a low-level, recurring exposure to a carcinogen is more hazardous than a single exposure to the same amount.

The present investigations confirm previously reported observations on the successful production of cancers in mice exposed to condensates of crude benzol extracts of the particulate phase of urban air pollutants (Leiter and Shear; Kotin and Falk; and others) and on the demonstration of 3,4-benzpyrene in such materials reported by several English and American workers (Waller; Stocks and Campbell; Kotin and Falk). These studies provide also additional evidence indicating that solvent fractions apparently free from 3,4-benzpyrene, also possess carcinogenic properties to mice (kotin and Falk). One of the new contributions of this exploratory methodologic approach to the problem of air pollutants and lung cancer hazards is represented by the demonstration of distinct variations in the quantitative and qualitative composition of air pollutants from different cities as reflected by the solvent fractions studied and by the observation of distinct discrepancies in the carcinogenic responses obtained by a repeated subcutaneous administration of standardized amounts of these fractions. If, therefore, the assumption is correct that these constituents of the total spectrum of atmospheric carcinogenic air pollutants are responsible for a still undetermined part of the lung cancers developing among urban populations, it is obvious that the share which any particular fraction has in such cancerous reactions most likely would vary in different cities and would depend upon the degree to which the individual fractions contribute to the total air pollution.

Major Findings (cont):

1. Bioassays performed on mice with four subcutaneously injected solvent fractions of the particulate phase of air pollutants of eight cities containing aromatic as well as aliphatic hydrocarbons, yielded cancerous reactions with all four fractions.

b. (cont)
Major Findings (cont)

2. The cancer yield obtained by the various fractions differed with the fraction as well as with the city from which the air pollutants were collected.

3. With the exception of air pollutants of Birmingham, which showed the highest total cancer yield and which had also the highest amount of 3,4-benzpyrene, there was no consistent correlation between the degree of carcinogenic potency of the solvent fractions bioassayed, the amount of 3,4-benzpyrene, and the reported lung cancer mortality rate in the eight studies studied.

4. It appears that every community has an atmospheric carcinogenic spectrum which depends upon local conditions which include not only industrial activities and traffic conditions, but also climate, solarization, and geologic conditions.

5. An exploration of the relative role and importance of these factors for the lung cancer panorama of individual cities should include an investigation not only of the aromatic and aliphatic hydrocarbons present in the particulate phase of air pollutants but also of metallic, mineralic and radioactive constituents as well as the relative chemical stability and the physical status of some of these elements in the atmosphere.

6. The present studies represent merely a first and preliminary step in a prolonged and comprehensive program dealing with relationship between air pollutants and health hazards, including cancers.

Significance: - - The significance of these studies was discussed in detail in a joint report presented before the meeting of the Air Pollution Control Association in May 1960. The findings available at that time strongly suggest that the marked differences in lung cancer rates observed among the various metropolitan areas analyzed by the Air Pollution Program are at least, in part due to distinct differences in the quantitative and qualitative composition of the spectrum of air pollutants present in individual cities. The observations made, moreover, indicate that urban air pollutants from various sources represent one of the major sources of lung cancer hazards.

b. (cont)

Proposed Course: - In an attempt to improve the tumor yield of the fractions of air pollutants the crude benzol fraction of the eight cities is being injected once a month into the right pleural cavity of mice and rats. It is hoped that through this procedure cancers of the lung may be obtained and thereby proof of a carcinogenic effect of chemicals present in the fraction upon pulmonary tissue will be demonstrated. An expansion of the present experiments on cancer hazards from air pollutants must wait until the bulk of the animals used in these experiments have died.

PART B INCLUDED - - - YES

PART B:

Publications:

a-1 and a-2: Hueper, W. C. and Payne, Wm. W.: Carcinogenic Studies on Petroleum Asphalts, Cooling Oil, and Coal Tar. Arch. Pathol. 70:372-384, 1960.

a-3: Hueper, W. C. and Payne, Wm. W.: Carcinogenic Studies on Soot of Coffee-Roasting Plants. Arch. Pathol. 69: 716-727, 1960.

b.: Hueper, W. C., Kotin, P., Tabor, E. C., Payne, Wm. W., Falk, H. and Sawicki, E.: Carcinogenic Bioassays on Air Pollutants. Distributed as Multiliths to members of the Air Pollution Control Association and upon request by the Medical Air Pollution Program, U. S. P. H. S.

Hueper, W. C.: Air Pollution and Lung Cancer. To be published in a Japanese Medical Journal (Kyushu University).

Hueper, W. C.: Luftverunreinigungen und Krebs. Proc. 5th Session, Commission for Occupational Cancers, German Research Council, Bad Godesberg, March 1959.

Payne, Wm. W. and Hueper, W. C.: The Carcinogenic Effects of Single and Repeated Doses of 3,4-benzpyrene. Am. Industr. Hyg. Assoc. J. 21:350-355, (October) 1960.

Serial No. NCI-211 (a & b)
1. OADR
2. Environmental Cancer
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: - Epidemiological Studies of Environmental Carcinogens

a. Epidemiological Study of Lung Cancer in Workers Exposed to the Inhalation of Zinc Chromate Paint in Airplane Construction and Maintenance Plants.

Principal Investigator - Wm. W. Payne
Other Investigator - - - W. C. Hueper

Cooperating Units: - - - U. S. Air Force and a private airplane manufacturer.

b. Epidemiological Study of Lung Cancer and Asbestosis in Workers Exposed to Asbestos in Shipyards.

Principal Investigator - Wm. W. Payne
Other Investigator - - W. C. Hueper

Man Years (calendar Year 1960)
Total: --------- 0.8
Professional: ---- 0.2
Other: -------- 0.6

Objectives: - - Epidemiological studies have shown that workers in chromate-producing plants have an abnormally high incidence of cancer of the lung. Observations in Europe suggest that workers exposed to chromium pigments also experience a respiratory cancer hazard, but epidemiological evidence is lacking.

The airplane manufacturing and maintenance industry appears to offer the best opportunity for studying workers who have been exposed to zinc chromate paints and related compounds. The Office of the Surgeon General and the Air Materiel Command of the Air Force, and the director of Health

a. (cont)

and Safety of a large airplane manufacture have expressed and shown a willingness to cooperate in this study which is designed to determine whether exposure to zinc chromate paint by inhalation results in an increased risk to lung cancer.

Methods Employed: - - From the personnel records of two large Air Force maintenance bases and one private airplane construction plant, the names, social security number, date of birth, approximate length of service were obtained for another group of workers in the same plant who would have a minimal exposure to zinc chromate. Date of birth and length of employment was matched as closely as possible.

From the Social Security Administration it was learned whether a death claim had been filed for each of these workers and if so, in which State. Using the available information, copies of death certificates have been obtained from the several State Health Departments. Where any form of cancer is specified as the cause of death, an effort will be made to determine the basis on which the diagnosis was made. This will be done for both painters and control group. An analysis will be made to determine whether there is any significant difference in the prevalence of cancer in the two groups.

Major Findings: - - Copies of most of the death certificates have been obtained but no results will be available until an analysis can be made.

Significance: - - Since chromate paints are used so extensively, the results of the study would be of general public health interest. The investigation will afford an opportunity to increase our knowledge regarding the carcinogenicity of metals and will supplement information gained from laboratory experiments.

Proposed Course: - - With the cooperation of State and local authorities the basis for the diagnosis of cancer will be determined. An analysis will be made of causes of death in order to compare the experience among the painters with that of the control groups. If the numbers are not large enough to yield reliable results, the records will be kept for a few years and then the names will be sent to the Social Security

a. (cont)

Administration again to determine which workers have died in the intervening period. Again copies of additional death certificates can be obtained and another analysis made.

PART B INCLUDED:-----NO

b. Epidemiological Study of Lung Cancer and Asbestosis in Workers Exposed to Asbestos in Shipyards.

Objectives: - - Considerable evidence has been published associating asbestosis and cancer of the lung among workers exposed to asbestos. In a recent publication, however, two investigators claim to have demonstrated that a group of asbestos workers in Canada have not experienced an excessive liability to lung cancer. Consequently it is desirable that wherever possible the experience of other groups of asbestos workers should be examined to determine whether there is any relation between asbestosis and cancer of the lung.

Methods Employed: - - The names and other identification have been obtained for a group of federal workers who have been exposed to asbestos dust in the course of their employment as pipe coverers and insulators. None of these are employed presently at the above installation, and a number of them are known to be deceased. Further employment records will be obtained from Civil Service Records and then through the Federal retirement system or Social Security, the names of those who are dead will be obtained. From the death certificates the assigned cause of death will be determined. If this preliminary investigation suggests that there is a positive correlation between asbestosis and lung cancer, the study will be extended to include a larger group of workers employed in this trade. At the same time similar information would be obtained for a group of controls.

Major Findings: - - No work has been done except to obtain the names of the workers.

Significance: - - Since asbestos finds a wide variety of uses in industry and the building trades, it is important to ascertain whether

b. (cont)

there is an increased liability to cancer of the lung following the inhalation of this material.

Proposed Course: - - After additional information is obtained from Civil Service records, copies of death certificates will be obtained. If analysis of the data indicates there is a positive correlation, a larger group of workers will be incorporated into the study.

PART B INCLUDED----NO

(Note: - Because of the preliminary nature of this investigation, a summary Bio-Sciences Information Exchange, is not included).

Serial No. NCI-212 (1, 2, 3, 4)
1. OADR
2. Environmental Cancer
3. Bethesda, Md.

PHS-NIH
Individual Project Report
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PART A:

Project Title : - - Experimental Carcinogenesis of the Upper Digestive Tract

1, 2 and 3 - Effects of Various Chemicals on the Gastric Mucosa of Mice ("Seirogan", Benzo(a)pyrene, dibenz(a)anthracene)

4. Chemical Analysis of Whiskies for Aromatic Polycyclic Hydrocarbons

Principal Investigator - - M. Kuratsune
Other Investigator - - - - W. C. Hueper

Man Years (calendar year 1960)
Total: ---------- 3.1
Professional: ----- 1.0
Other: ---------- 2.1

1, 2 and 3:

Objectives: - - Cancer of the stomach is a rather frequent and often fatal human disease, which in Japan, Chile, and Finland accounts for over 50% of all cancer deaths among males. Attempts to produce gastric cancers in mice by feeding some of the known and potent polycyclic aromatic hydrocarbons have failed, although cancers of the glandular stomach can be obtained by a direct injection of such chemicals into the gastric mucosa. As a preliminary step in a comprehensive experimental attack on the gastric cancer problem, it appeared necessary to determine, if possible, the minimal effective dose of such chemicals which are constituents of the ordinary human diet and to investigate various factors which might favor or hinder their penetration into the esophageal and gastric mucosa when given orally. "Seirogan" is a brand of wood creosote extensively consumed in Japan as a protective against intestinal infections.

Methods Employed: - Thirty eight C-57 Black male mice were each injected with 16.1γ of benzo(a)pyrene dissolved in tricaprylin at the antrum of the stomach. Similarly, 41 mice and 46 mice of the same strain and sex were each injected with 1.61γ and 0.161γ of benzo(a)pyrene, respectively. The animals which were injected with 0.161γ of benzpyrene were again injected with 0.161γ of benzpyrene at approximately the same

Methods Employed (cont):

site as that of the first injection 6 to 7 weeks after the first injection. Two groups of mice of the same strain and sex were injected with 6.3γ and 0.63γ of dibenz(a,h)anthracene in tricaprylin at the antrum of the stomach. Another group of 40 mice were injected with 1.705γ of "Seirogan" extract in the same way. As controls, 41 mice of the same strain and sex were injected with the same amount of tricaprylin as that injected in the experimental groups.

Penetration of polycyclic aromatic hydrocarbons into the gastric mucosa and the esophageal epithelium were studied with the use of the following techniques: - - Various solutions of benzo(a)pyrene, benz(a)-anthracene, and fluoranthene were injected into the esophagus of mice through a polyethylene tubing. The injection was carried out very slowly, taking 60 seconds to complete it. Fifteen to 120 minutes after the injection the animals were sacrificed and the esophagus and stomach were excised. The stomach was opened, rinsed with a saline solution, then fixed in formalin solution. The esophagus was fixed without rinsing it. After 30 to 120 minutes' fixation, the tissues were frozen and cut. The cut specimens were irradiated by ultraviolet light and the fluorescence generated was examined by a microscope. The solutions of the hydrocarbons examined are as follows:

Saturated solution of benzpyrene in 20% ethanol
Ditto 40% "
Ditto 50% "
Ditto 60% "
Saturated solution of fluoranthene 50% "
Saturated solution of benz(a)-anthracene in 50% "
Ditto 60% "
Saturated solution of benzpyrene in 1% Caffeine aqueous solution
Saturated solution of fluoranthene in 1% " " "
Saturated solution of benz(a)-anthracene in 1% " " "

A benzpyrene solution in olive oil or tricaprylin - the concentration of which is the same as that of the saturated solution of benzpyrene in 50% ethanol - was also used.

Major Findings: - - After more than 10 months following an injection of the three chemicals into the gastric mucosa, no tumors have been observed. The penetration experiments yielded the following results:

Major Findings (cont):

1) Benzpyrene dissolved in oil enters the squamous epithelium of the esophagus and the forestomach, but not the mucosa of the glandular stomach.

2) Benzpyrene dissolved in ethanol solutions enters the epithelium of the forestomach and the esophagus, but not the glandular mucosa of the stomach.

3) Benzpyrene in ethanol solutions penetrates the esophageal epithelium more readily than when it is dissolved in olive oil.

4) Benzpyrene dissolved in a caffeine solution penetrates the epithelium of the esophagus and forestomach and also the glandular mucosa of the stomach. The penetration into the glandular mucosa is, however, less marked than that into the esophagus and forestomach.

5) Fluoranthene in 50% ethanol enters not only the esophagus and the forestomach, but also the glandular stomach.

6) Fluoranthene in 1% aqueous solution of caffeine readily enters the esophagus, the forestomach, and the glandular stomach.

7) Since the fluorescence of benz(a)anthracene in the solvent vehicles used is very weak, the observations of this compound were not conclusive.

Proposed Course: - - Since the principal investigator has returned to Japan, the surviving animals will be observed for a total of two years and will then be sacrificed for histologic study. The final results will be reported after these data have become available. The experiment then will be terminated.

PART B INCLUDED: - - - - -NO

4. Chemical Analyses of Whiskies for Aromatic Polycyclic Hydrocarbons.

Objectives: - - Epidemiologic studies concerning the relative frequency of cancers of the upper alimentary tract carried out in several countries in past decades have demonstrated an excessive liability of members of the liquor trade and of heavy consumers of hard liquors to cancers of the mouth and esophagus. Because of the rather high frequency of these cancers and their relatively bad prognosis, it was pertinent to ascertain, if possible, a specific causal basis for this statistical relation. Since American whiskies are aged in charred wooden barrels, while Scotch whiskies are processed with smoke for producing a special flavor, the

Objectives (cont)

suspicion arose that contaminants of carcinogenic nature might be introduced into whiskies from such sources which might account for the statistical association to cancers of the alimentary tract mentioned. A chemical analytical study as well as a bioassay investigation of extracts of various whiskies was undertaken for obtaining some factual evidence.

Methods Employed: - - Five different bourbons and 8 Scotch whiskies were extracted with methylene chloride. The extracts were subjected to a series of chromatographic fractionations and to spectrophotometric analysis by absorption spectra for demonstrating the presence of aromatic polycyclic hydrocarbons. The residue from evaporated "Old Forrester" was mixed with hexadecyl palmitate and implanted as a pellet into the subcutaneous tissue of the nape of the neck of mice. Each pellet contained about 8 mgm. of the residue.

Major Findings: - The results obtained are shown in table 1. Benzo(a) - pyrene and Benz(a)anthracene were not found in any specimen. The bioassay experiment has not yielded any tumors so far.

Table 1

Whisky	Proof	Phenanthrene, γ/l	Pyrene γ/l	Fluoranthene γ/l
Old Forester	86	7	---	Trace
J. W. Dant	100	5	0	Trace
Old Kentucky Tavem	100	7	Trace	0.8
Old Taylor	100	6	0	0
Old Hickory	86	2	Trace	0.5
Black & White	86.8	3	1	2
Vat 69	86.8	11	2	4
Haig & Haig	86.8	3	0.9	1
Johnnie Walker (Black)	86.8	Trace	2	Trace
Johnnie Walker(Red)	86.8	6	2	2
Holt's Buff Label	86.8	Trace	0.6	1
Loch Corrie	86.8	Trace	0.9	2
Glenlogie	86.8	Trace	0.4	Trace

Significance: - - Unless the future results of the bioassays should indicate the presence of carcinogenic properties in whiskies, the presently available observations do not incriminate any known polycyclic aromatic hydrocarbons in this respect. The statistical relation between an excessive consumption of hard liquor and cancers of the alimentary tract, therefore, may be associated with effects of alcohol on the mucosa

Significance: (cont)

of this organ system which are not carcinogenic by themselves but which merely favor the action or penetration of such agents upon the mucosal cells.

Proposed Course: - -The surviving mice will be observed for a total of 2 years and will then be sacrificed for histologic study. Since the principal investigator has returned to Japan, this experiment will then be terminated.

PART B INCLUDED ------NO

Serial No. NCI-213
1. OADR
2. Environmental Cancer
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
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PART A:

Project Title: - Experimental Studies on Carbon Adsorbates of Raw and Processed Water.

Principal Investigator - W. C. Hueper

Man Years (calendar year 1960)
Total: ---------- 0.4
Professional: ------ 0.1
Other: ---------- 0.3

Objectives: - - Eluates of carbon adsorbates from raw and processed river water obtained at Nitro, West Virginia, by investigators of the R. A. Taft Sanitary Engineering Center are being tested for carcinogenic properties so as to obtain information whether or not the industrial and human use of such waters might constitute a cancer hazard to the human population.

This preliminary investigation which in fact is a continuation of similar studies conducted several years ago on similar materials, represents the first step of a large-scale exploration of water supplies of various sources for cancer producing chemicals to which the general population has as intimate a contact as with carcinogenic air pollutants.

Methods Employed: - - The material on hand is injected intrapleurally into rats and mice, is painted on the skin of mice and is incorporated into the food of mice.

Major Findings: - - Several cancers have been observed in mice following a monthly subcutaneous injection of adsorbates of raw water. Since the experiment is still in an early stage, additional cancerous responses may appear later among the survivors.

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Significance: - - The increasing contamination of public water supplies with industrial wastes containing carcinogenic chemicals and radioactive materials constitutes a growing danger to the health of the American population and deserves the serious interest of public health.

Proposed Course: - - Continuation of the present investigations during the coming year.

PART B INCLUDED: ---------NO

Serial No. NCI-301
1. Lab. of Biochemistry
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
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Part A.

Project Title: Secretary Duties in the Laboratory of Biochemistry.

Principal Investigators: A. E. Stotler and J. K. Shores

Other Investigators: P. K. Baylin

Cooperating Units: None

Man Years (calendar year 1960):
Total: 3
Professional: 0
Other: 3

Project Description:

Objectives: The objectives of the Secretarial Unit in the Office of the Chief are to: Maintain personnel, correspondence, reprint and requisition files, maintain leave, overtime, and budget records, maintain a Laboratory library, arrange for travel of professional staff, compose and type letters, memoranda, requisitions, and shop orders, and type manuscripts, lantern slides, travel requests, personnel requests, and miscellaneous charts and articles. The above duties are performed for 31 professional and 29 subprofessional employees.

Methods: Since January 1960, this Unit has typed the following: Approx. 1500 pages original manuscript, 950 letters and memoranda, 50 lantern slides, 500 pages of miscellaneous material, 1500 requisitions, 300 instrument shop orders, 200 Request for Services, 100 reports of surplus property, 50 Shipment Requests, 70 Travel Requests, and 40 Personnel Action Requests.

In the future this Unit will continue to do its utmost to produce as much work as possible and to perform this work as accurately and rapidly as is possible.

Part B included: NO.

Serial No. NCI-302
1. Lab. of Biochemistry
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Development of micro and ultramicrochemical methods for the analysis of biological samples arising from a basic examination of the neoplastic processes.

Routine microchemical empirical analysis.
Routine biochemical analyses.
Basic investigation into the Infrared and Ultraviolet absorption of proteins, oligopeptides, dipeptides, amino acids and nucleic acids.

Principal Investigator: Robert J. Koegel

Other Investigators: J. Williams, I. Losonczy

Cooperating Units: None

Man Years (calendar year 1960):
Total: 2-3/4
Professional: 1
Other: 1-3/4

Project Description:

The microchemical unit provides a versatile and complimentary analytical service functioning in direct collaboration with the independent investigators of the Biochemistry Laboratory. Operationally the unit strives to make available on a service basis the most current procedures in the area of micro and ultramicrobiochemical analysis.

The routine service of the unit continues as described in previous annual reports, however, during 1960 the character of the requested work load changed significantly in a qualitative but not a quantitative way. Specifically the total number of microchemical empirical analyses CH, N, Cl, S, dry weight, etc., became a small fraction of the total

Part B included: NO

analyses performed. Quantitatively there were some 5,500 individual analysis completed during the 1960 Calendar Year and this figure reflects a service workload performance equal to that of 1959 executed with twenty percent decrease in staff.

In addition to the service work described in the previous paragraph, the unit adapted from current chemical literature three ultramicro procedures which are now offered as a routine service.

1. A procedure for the determination of total phosphorous in the range of 0.25 μg. to 2.0 μg. with a precision and accuracy of ± 5.0%. This procedure with the supporting data on refractory organic phosphorous compounds is being prepared for publication early in January of 1961.

2. The ultramicro methods for the determination of whole blood or plasma oxygen and carbon dioxide using the classical Van Slyke procedures and the Natelson Manometric apparatus.

3. Procedures for the determination of Na^+; K^+, Ca^{++}, Mg^{++}, Cl and total phosphorous on serum samples in the range 0.275 ml. to 0.300 ml. The accuracy and precision for each constituent determined being ± 7.0%.

No work was accomplished during 1960 on the projected Infrared Absorption Program. It is hoped that this area will be actively utilized during 1961.

Serial No. NCI-306
1. Lab. of Biochemistry
2. Phys. Chem. and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Studies on hydroxyamino acids

Principal Investigators: Theodore T. Otani, Nobuo Izumiya, Sanford M. Birnbaum and Milton Winitz

Other Investigators: None

Cooperating Units: None

Man Years (Calendar Year 1960):
Total: 4.2
Professional: 3.2
Other: 1

Project Description:

Studies under this project may be categorized as follows: (a) Chemical synthesis and enzymic resolution of a series of β-hydroxyamino acids, as well as the preparation of certain N-acyl derivatives thereof, which may prove of biochemical and/or oncological interest.

(b) Study of these compounds with regard to their potential worth as growth inhibitors in different microbiological systems selected primarily on the basis of their utility for investigations of amino acid metabolism and as rapid screening devices for possible anti-tumor effects.

(c) The development of a convenient method of synthesis and resolution of δ-hydroxylysine.

Methods: (a) Synthesis, resolution and N-acyl derivatives of α-aminoβ-hydroxy acids. The previous annual report described the synthesis of a series of α-amino-β-hydroxy acids in a single step reaction which involved the treatment of an alkaline solution of an amino acid with an aldehyde, in the presence of catalytic amounts of cupric ion, according

Part B included: YES

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to the following scheme:

$$RCHO + NH_2CHR'CO_2H \xrightarrow[OH^-]{Cu^{+2}} RCH(OH)CR'(NH_2)CO_2H$$

Such reaction scheme has been extended, with suitable modifications, to include the synthesis of β-phenylserine and p-nitro-β-phenylserine from the interaction of glycine with benzaldehyde and p-nitrobenzaldehyde, respectively, under the above described conditions. Like attempts to utilize o-, m- or p-hydroxybenzaldehyde, p-methoxybenzaldehyde or p-aminobenzaldehyde as the aldehyde reactant did not yield the desired products in satisfactory amount. However, comparable use of n-butyraldehyde did permit the isolation of β-hydroxynorleucine in satisfactory yield. Hence, the β-hydroxyamino acids synthesized by this procedure thus far include serine, threonone, allo-threonine, α-(hydroxymethyl)-serine, α-methylserine, α-ethylserine, β-hydroxyleucine, β-hydroxyaspartic acid, β-hydroxynorleucine, β-phenylserine, and p-nitro-β-phenylserine. Separation of the diastereomers of the synthetic threonine-allothreonine mixture was achieved on an Amberlite CG-120 column, the eluant being ammonium acetate buffer at pH 6.3. N-Acyl derivatives of the aforementioned compounds include N-chloroacetyl- and N-dichloroacetyl-α-methylserine, N-chloroacetyl-α-ethylserine and N-dichloroacetylthreonine.

Attempts were made to resolve α-methyl-DL-serine and α-ethyl-DL-serine via their N-chloroacetyl derivatives. An unexpected and interesting result was observed in the resolution of the former amino acid. The hydrolysis, instead of stopping at the expected 50 percent point, as is expected for a racemic mixture, continued beyond that point and approached 100% hydrolysis. Upon separation, the product of hydrolysis was found to be completely racemic, thereby indicating that the acyl derivatives of both optical forms were attacked by the enzyme. This is of particular interest in that the hydroxymethyl and the methyl groups are apparently so sufficiently similar as to markedly lessen the ability of the enzyme to discriminate between the assymetry of both optical isomers of the molecule. This finding should prove of interest in the study of the mechanism of enzyme action.

α-Methylserine could, however, be partially resolved by terminating enzymic action at slightly beyond the 50% hydrolysis point. By this means, a D-isomer with a specific rotation ($[\alpha]_D$ = + 6.5° in water) higher than any hitherto reported has

been obtained. The specific rotation of the L-isomer ($[\alpha]_D$ = -3.0° in water), however, indicated appreciable contamination with the D-antipode. α-Ethylserine could be resolved in more satisfactory manner to yield specific rotation values, in water of $[\alpha]_D$ =+3.8° and $[\alpha]_D$= -3.8° for the L- and D-isomer, respectively.

(b) Growth inhibition studies. - These studies were undertaken with the purpose of investigating the potential antimetabolic properties of the compounds under consideration, with particular emphasis as to their anti-tumor character, by using a carefully selected group of microorganisms as a screening device. In order to ascertain whether or not any of these compounds would serve as an antimetabolite, an assay system was first set up using E. coli (ATCC No. 9637) as the microorganism of choice since this organism is capable of synthesizing all essential nutrilites from a relatively simple culture medium; its growth should therefore represent an exceptionally large array of metabolic steps. The results obtained with this microorganism are listed as follows:

	50% inhibition γ/ml assay medium:
α-(Hydroxymethyl)-DL-serine	0
α-Methyl-DL-serine	0
α-Ethyl-DL-serine	0
α-Ethyl-L-serine	0
α-Ethyl-D-serine	0
β-Hydroxy-DL-leucine	6.3
β-Hydroxy-DL-aspartic acid	2.3
β-Nitro-DL-β-phenylserine	0
N-Chloroacetyl-α-methyl-DL-serine	0
N-Dichloroacetyl-DL-serine	0
β-Hydroxy-β-methylaspartic acid	117
γ-Hydroxyglutamic acid (isomer A)	slight at 137
γ-Hydroxyglutamic acid (isomer B)	53
L-O-Methylthreonine	1.1
L-O-Methyl-allo-threonine	137

The anti-tumor screening assays are now in progress and the results will be presented in the next report.

(c) Synthesis and resolution of δ-hydroxylysine. - The synthesis of δ-hydroxylysine was achieved via the following reaction

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2. Phys. Chem. and Chromatography Section

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scheme:

$$CH_2 = CHCHO + CH_3CONHCH(CO_2Et)_2 \longrightarrow HOCCH_2CH_2CNHCOCH_3(CO_2Et)_2$$

$$\xrightarrow{CH_3NO_2} CH_2(NO_2)\ CH(OH)CH_2CH_2CNHCOCH_3(CO_2Et)_2 \xrightarrow[H+]{H_2,Pd}$$

$$CH_2(NH_2)CH(OH)\ CH_2CH_2CH(NH_2)CO_2H.$$

This reaction scheme which is a modification of that reported in the literature (VanZyl, Van Tamelon and Zuidema, J. Am. Chem. Soc., 73, 1765 (1951), permitted the isolation of an epimeric mixture of δ-hydroxylysine in about 50% overall yield. This yield is some 2-3 times greater than any hitherto reported. Separation of the diastereomers was achieved by fractional crystallization of their α-chloroacetyl-ε-carbobenzoxy derivatives. Resolution of the latter derivatives was accomplished enzymically using cobalt-activated acylase I. Use of cobalt ion in the enzymic digested permitted the resolution to go to completion in about 3 days instead of the 2-3 weeks generally required for this resolution in the absence of cobalt ion. The 4 optical antipodes of δ-hydroxylysine so procured exhibited optical purities and specific rotation values in agreement with those reported in the literature.

Significance: The β-hydroxyamino acids may conceivably play a role (1) in studies of amino acid metabolism in microbiological systems by acting as inhibitors of enzymes involved in specific steps of the intermediary metabolic pathways; (2 in the study of anti-tumor agents, as their N-acyl derivatives, (3) in the study of mechanism of enzymic action, (4) in the study of new pathways of amino acid synthesis, such as those cases where the β-hydroxyamino acid may support instead of inhibit growth, and (5) perhaps, because of their facile formation under near physiological conditions, in the study of possible non-enzymatic in vivo synthesis of certain essential components.

Proposed Course of investigation: (1) In regard to synthesis of β-hydroxyamino acids, it might be of interest to prepare ring halogenated or ring alkylated β-phenylserines as analogs

of phenylalanine or of tyrosine. It should be of interest too, to ascertain whether thioaldehydes could participate in similar reactions to form β-thiol-amino acids, for example, preparing β-thiol-α-amino butyric acid (β-thio-L-threonine) from thio-acetaldehyde, or β-phenylthioserine from thiobenzaldehyde and glycine, and cysteine from thioformaldehyde and glycine, etc.

(2) N-Acetylated derivatives, and the N-chloroacetyl- and N-dichloro-acetyl derivatives are to be prepared, both for the purpose of resolution as well as for studies as possible anti-tumor agents.

(3) The anti-tumor screenings using four microbiological systems are now under way.

(4) If any of these compounds prepared shows promise as anti-tumor agents, these will be assayed using mammalian cell systems and, with further promise, using tumor-bearing animals.

(5) Resolution of the racemic mixtures of these β-hydroxyamino acid will be continued.

(6) Separation of the diastereoisomers, especially of those compounds found to be active inhibitors in the microbiological assay, will be attempted. The diastereomers of certain β-phenylserines show good separation on paper chromatography and appear to be capable of being separated by the column technique.

(7) The interesting enzymic behavior of α-methylserine as a symmetrical compound will be studied further.

(8) There is evidence that serine and threonine are attacked by the same dehydrase, serine dehydrase. Having a series of the higher homologs of the β-hydroxyamino acids, we are in, an excellent position to determine whether or not serine dehydrase is a general β-hydroxyamino acid dehydrase. This work is now in progress.

(9) In view of the powerful inhibitory effect exhibited by L-O- methylthreonine, and in view of the fact that azaserine

is an O-acylated serine, it will be interesting to synthesize O-acetylated derivative of these β-hydroxyamino acids and study their potential anti-metabolic, and anti-tumor properties.

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Part B.

Honors, Awards and Publications:

Publications other than abstracts from this project:

(1) Otani, T. T., And Winitz, M.: Studies on Hydroxyamino Acids. I. Synthesis of some α-alkylated Serines. Arch. Biochem. Biophys. 90, 254-259, 1960.

(2) Otani, T.T. Two-Dimensional Paper Chromatography. The Science Teacher 27, 29-31, 1960.

(3) Greenstein, J. P., Izumiya, N., Birnbaum, S. M., and Winitz, M. (checkers): Hydroxy-L-lysine, Hydroxy-D-lysine, Allohydroxy-L-lysine and Allohydroxy-D-lysine (submitted by W. S. Fones). Biochemical Preparations, in press.

(4) Winitz, M., Birnbaum, S. M. and Greenstein, J. P.: L- and D-threo-β-Methylaspartic. Biochemical Preparations, in press.

(5) Winitz, M. and Birnbaum, S. M. (Checkers): β-Methylaspartic Acid (submitted by Barker, H. A. and Wawszkiewicz, E.J.). Biochemical Preparations, in press.

Serial No. NCI-313
1. Lab. of Biochemistry
2. Physical Chemistry and Chromatography Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Chromatographic Analysis of Protein Components of Serum in Health and Disease.

Principal Investigators: E. A. Peterson and H. A. Sober

Other Investigators: A. Martinez Canaveri (Visiting Scientist)
G. Casillas (Visiting Scientist) B. L. Vallee

Cooperating Units: Academia Nacional de Medicina de Buenos Aires, Argentina
Peter Bent Brigham Hospital, Boston, Massachusetts

Man Years (calendar year 1960)
Total: 1-1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To develop and apply chromatographic procedures to the isolation and identification of the many macromolecular components in normal and pathological serum and plasma.

Methods Employed: Plasma, serum, and their preparations are subjected to chromatographic fractionation on cellulosic ion exchangers, using gradient and stepwise elution techniques. The resulting fractions are examined by physical, chemical, and immunochemical techniques, and specific biological activities are characterized.

Part B included: YES

(ATTACHMENT I)
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Major Findings: 1. Blomback FI-O preparations of Factor VIII (antihemophilic factor) have been purified by chromatography on DEAE-cellulose, with good recovery of this factor in purer form than any reported in the literature. Decreased stability of the eluted activity toward storage and freezing has, to a considerable extent, been overcome by the addition of glucose. Chromatographic evidence suggests that additional purification can be achieved by further development of the method.

Factor VIII activity can also be adsorbed on DEAE-cellulose directly from undialyzed, undiluted plasma from which prothrombin and other substances that tend to inactivate Factor VIII have been removed by adsorption on aluminum hydroxide. The Factor VIII can then be recovered by elution from the DEAE-cellulose with NaCl. Although this work is at a preliminary stage, it gives promise of providing Factor VIII preparations suitable for clinical use.

2. The chromatography of crystalline bovine plasma albumin on DEAE-cellulose with a suitable gradient yields a skewed peak, suggesting heterogeneity. Treatment of the BPA with cetyltrimethylammonium bromide (CTAB) before chromatography preferentially converts the earlier, major portion of the peak into complexes having little or no affinity for the adsorbent. As the major component is thus diminished, the residual, presumably unaltered, albumin can be clearly seen to comprise at least four components. Higher concentrations of the reagent convert the later peaks as well. Unlike the CTAB complex, previously prepared from highly purified human serum mercaptalbumin, that formed with crystalline bovine albumin is largely dissociated by dialysis. The liberated protein occupies the same chromatographic position as the last component of untreated BPA and consists of dimer and higher polymers.

Chromatography of an old sample oflyophilized Red Cross human Fraction V gave two approximately equal peaks. The first was pure monomer, the second was 70 per cent dimer and trimer. Fresh Red Cross Fraction V, on the other hand appeared as a single, highly skewed peak containing little or no dimer.

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3. Quantitative determination of the esterase activity associated with crystalline bovine plasma albumin, using dichloroindophenyl acetate as substrate, showed all the chromatographic fractions to be equally active. The activity is therefore not attributable to an enzyme impurity. The addition of cetyltrimethylammonium bromide to the albumin caused a three-fold increase in esterase activity, although the same concentration of this compound had no effect on the substrate in the absence of albumin.

4. Attempts to quantitate the qualitative distribution of trace metals in serum protein previously reported have demonstrated the need for vigorous control against metal contamination. An eluting buffer, tris succinate, has been found to give satisfactory protein resolution and to be completely combustible in the spark gap used for trace metal analysis. Conversion from glass to plastic equipment has been accomplished thus avoiding a troublesome source of metal contamination.

Significance: 1. Purified Factor VIII is required for chemical studies regarding its structure and mechanism of action and for clinical use in the treatment of hemophilic patients.

2. Changes in the chromatographic behavior of the serum proteins must be well understood if the chromatographic patterns are to be interpreted with confidence. Albumin is the major protein component in serum, and it appears to be the most susceptible to alterations of this kind, so study of these effects in albumin are particularly important. Information already acquired has shed light on the nature of the heterogeneity of albumin as observed in serum chromatograms which may reflect its transport function. See also NCI-357.

3. The esterase activity of highly purified albumin, first demonstrated by Kramer, may represent a true metabolic function of the albumin, or it may be a fortuitous consequence of the structure of the molecule. In the latter case it would be a particularly interesting subject for the study of the mechanisms of enzyme action, especially since a pseudo-coenzyme activity has been found in a substance known to form a complex with it.

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4. A variety of protein - metal combinations in serum still remain unrecognized and unknown. Their function and structure remain unknown. With the well-known participation of metals in a great number of enzymatic systems, it would seem that the circulating metal proteins of serum should be studied as potential transport, storage or enzymatic agents.

Proposed Course of the Project: 1. Further purification of Factor VIII will be attempted by modification of the present procedure and by rechromatography. More effective stabilization of the activity will be sought. Methods suitable for the isolation of Factor VIII directly from undialyzed, undiluted plasma on a large scale will be investigated.

2. The complexes obtained by combining albumin with cationic and anionic lipids will be further studied by chromatography, electrophoresis, and sedimentation. Continued attempts will be made to prepare albumin free of all lipid.

3. The mechanism whereby esterase activity in purified albumin is increased by the addition of cetyltrimethylammonium bromide will be investigated in more detail. Similar effects by other lipids (cationic, anionic, and nonionic) will be looked for.

4. Studies aimed at the recognition, purification and characterization of the metal-protein components of serum will be continued.

(ATTACHMENT IV)
Serial No. NCI-313

PHS-NIH
Individual Project Report
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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Peterson, E. A. and Sober, H. A.: Chromatography of the Serum Proteins. Plasma Proteins, edited by F. W. Putnam. Academic Press, New York, 1960. This paper was listed as "in press" in the 1959 report.

2. Levy, H. B. and Sober, H. A.: A simple chromatographic method for preparation of gamma globulin. Proc. Soc. Exp. Biol. and Med. 103: 250-252, 1960.

Serial No. NCI-335
1. Lab. of Biochem.
2. Phys. Chem. and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Quantitative Nutritional Studies with Water-Soluble, Chemically Defined Diets.

Principal Investigators: M. Winitz, S. M. Birnbaum and M. C. Otey

Other Investigators: T. Sugimura (Visiting Scientist)

Cooperating Units: C. I. Jarowski and A. Puccini (both of Chas. Pfizer and Co., New York).

Man Years (Calendar Year 1960):
Total: 7
Professional: 2
Other: 5

Project Description:

Objectives: The previous annual report dealing with this project was concerned with the formulation of a complete and nutritionally adequate water-soluble, chemically defined diet composed of 18 crystalline L-amino acids (except for glycine), the water-soluble vitamins, the requisite salts, glucose, the fat-soluble vitamins, and ethyl linoleate as the source of essential fatty acid. Description was also given of the successful application of such chemically defined diets to the study of specific enzyme systems and metabolic processes *in vivo*. Extension of these studies with chemically defined diets has included the following:

(a) the formulation of a water-soluble chemically defined diet which would incorporate the minimal amounts of essential and non-essential amino acids consistent with maximal growth of rats;

(b) the use of chemically defined diets in studies on the *in vivo* utilization of D-amino acids;

Part B included YES

(c) the effect of water-intake on growth of animals fed chemically defined diets;

(d) the use of chemically defined diets to test a unique concept, proposed by Dr. C. I. Jarowski of Chas. Pfizer and Co., that the ideal essential amino acid requirements for any animal species may be formulated on the basis of the amino acid levels in the blood plasma of that particular species, in the fasting state.

Major Findings: (a) In the execution of quantitative in vivo metabolic studies with a given animal species, the employment of a balanced diet, wherein each and every component is known and chemically-defined is a necessary prerequisite to the procurement of unequivocal data. An excess or deficiency of a single essential component of the diet might easily cause a disruption of the normal metabolic processes and thus might well mask or otherwise alter the response to the single variable under investigation. The study described directly below was therefore devised in order to determine the minimal level at which each of the dietary amino acids had to be provided in the diet commensurate with the needs for maximal growth.

Earlier studies have revealed that chemically-defined diets, composed of the ten essential L-amino acids (in levels recommended by Rose, et al.), 8 non-essential L-amino acids, the requisite vitamins and salts, glucose and ethyl linoleate, when provided to rats ad libitum as 50% solutions in water, sufficed to meet the nutritive requirements for growth, lactation and reproduction over several generations. In the attempt to develop a diet wherein each of the essential L-amino acids was present at the minimal level consistent with optimal growth, diets were prepared which incorporated each of the essential amino acids (taken one at a time) in an amount ranging from 0 to 120% of that previously employed. By variation of the non-essential nitrogen level all mixtures were kept isonitrogenous but were otherwise identical. Such diets were fed to weanling male, Sprague-Dawley rats over a 10 day period and the minimal level of each essential amino acid commensurate with optimal growth ascertained. Minimal levels of the essential amino acids, relative to the levels in diets previously studied, were as follows: L-lysine, 60%; L-phenylalanine, 40%; L-tryptophan, 80%; L-isoleucine, 100%; L-methionine, 60%; L-leucine, 100%; L-valine, 80%; L-histidine, 60%; L-threonine, 100%. With these ratios as the basis, it became possible to

formulate chemically-defined diets whereon weanling rats grew at a rate of over 4 g. per day and with more efficient utilization of nitrogen than had been observed hitherto.

(b) Certain of the essential L-amino acids required by the rat may be effectively replaced by their respective D-antipodes. Such utilization of D-amino acids presumably arises from their successive conversion, first to the corresponding α-keto analogs by the action of D-amino acid oxidase, and thence to the L-antipodes by transamination:

D-Amino acid ⟶ α-keto acid ⟶ L-amino acid

Thus, for example, arginine, methionine, tryptophan and leucine may be replaced in whole or in part by the corresponding D-isomers in the diet of the rat. Earlier data from this Laboratory revealed that when D-methionine was employed instead of L-methionine in such diets, no change in growth response of weanling rats was observed over a 10 day period. However, D-tryptophan was only partially effective in replacing its corresponding L-antipode and D-leucine even less effective, since this latter amino acid had to be added at a level equivalent to 1.4 times its L-antipode before a significant growth response was observed. Now it should be noted that although D-leucine is converted by D-amino acid oxidase to the α-keto acid at an appreciably faster rate than is D-tryptophan to its corresponding α-keto acid *in vitro*, the degree of utilization of the latter amino acid *in vivo* exceeds that of the former amino acid. A comparable situation was found with D-alloisoleucine, which was reported in the literature to be completely ineffective as a replacement for L-isoleucine in the diet of the rat but which is, at the same time, very rapidly oxidized by D-amino acid oxidase *in vitro*. In order to ascertain why the ability of the organism to utilize the D-antipodes of the essential amino acids varies so considerably, and why results are sometimes obtained that are in variance with those that would be expected on the basis of *in vitro* enzymatic behavior, experiments were set up in which groups of animals were fed chemically defined diets wherein the L-isoleucine component was replaced, in three separate diets, with one, two and three equivalents, respectively, of D-alloisoleucine. Although no growth was observed with the first two diets, a marked growth response was noted with the third. The data therefore revealed that D-alloisoleucine could be utilized *in lieu* of L-isoleucine

if present in sufficiently high concentration in the diet. Examination of the urine of these animals also indicated that the D-alloisoleucine is excreted at an appreciably more rapid rate than is D-leucine, D-tryptophan or D-methionine and thus provided a plausible explanation for both the ability of D-alloisoleucine to support growth when provided in the diet in relatively high concentration and for the relatively lesser degree of utilization observed with D-alloisoleucine than with the other above-mentioned D-amino acids, despite its appreciable susceptibility to D-amino acid oxidase in vitro. In any event, the data conclusively revealed that the degree of utilization of D-amino acids by an animal organism is conditioned not only by the rate at which they are enzymically converted to their L-couterparts in vivo, but also by the extent of their renal resorption.

(c) Previous experiments in this Laboratory indicated that rats provided with chemically defined diets as 50% solutions in water will, in addition, ingest daily an amount of water (provided in separate water bottles)which varies in volume from about 15% to 150% of their dietary intake. In order to ascertain whether dietary intake would be influenced by the denial of water (other than that provided in the diet), two groups of weanling rats were set-up, one of which was provided the 50% diet solution in addition to an independent water source, and the other of which was provided the 50% diet solution alone. After six weeks, the average weight gain of animals in both groups (12 animals per group) was identical, as was their dietary intake. No differences in physical appearance, general well-being or activity between the groups were evident, although the first-mentioned group was provided with and actually did ingest appreciable amounts of water in addition to the diet solution. It would appear, therefore, that the amount of water in a 50% diet solution is adequate for normal growth and well-being of rats, for a limited time at least, in the absence of independently supplied water.

(d) An exhaustive survey of the nutritional literature has suggested that the free amino acid levels in the blood plasma of a given animal species, in the fasting state, might serve as a precise indicator of the dietary amino acid requirements of that species. Such concept would permit the facile formulation of diets, nutritionally adequate with respect to the essential amino acids, without resort to expensive and time-consuming growth or nitrogen balance studies. As a test of its

validity, studies were designed to compare the growth response of rats provided with the most efficient water-soluble, chemically defined diet hitherto devised in this Laboratory, with that of rats fed comparable diets wherein the essential amino acids were present in the same relative ratios as they appeared in the plasma of the fasting rat. The data unequivocally demonstrated that the former and latter diets elicited comparable growth rates, despite striking differences in their essential amino acid compositions. Thus, on the basis of the fasting amino acid plasma levels of the rat, the formulation of a nutritionally adequate diet could be achieved that was equally as efficient as the best chemically defined that could be devised using the classical approaches of nutrition. The far-reaching and profound implications in the nutritional field possessed by this very interesting and unique concept are discussed in the section below.

Significance: The applicability of water-soluble, chemically defined diets for use (a) in quantitative metabolic studies in man and in laboratory animals, (b) as food for germ free animals, (c) as a food supply in space travel, (d) in treatment of patients with metabolic functions, e.g. oligophrenia phenylpyruvica, (e) as food for patients with ulcerative colitis, (f) in parenteral feeding, (g) in treatment of kwashiorkor and (h) in the study of specific enzyme systems and metabolic processes in the whole animal organism, has been described in an earlier annual report. The present report embraces (a) the successful development of a water-soluble chemically defined diet which permits a better growth and is more efficiently utilized than any yet described, (b) the use of the diet in utilization studies of D-amino acids and the role of renal resorption in governing such utilization and (c) the demonstration that 50% aqueous diet solutions will supply not only all of the requisite nutritional components, but also adequate water to support normal growth and maintenance over a prolonged period in the absence of independently supplied water.

In addition, a study was designed which unequivocally demonstrated that chemically-defined diets, wherein the essential amino acid components are present in the same relative ratios as they appear in the blood plasma of the fasting rat, permitted the rapid formulation of a diet that was at least equally as efficient as the best chemically defined diet hitherto described in this Laboratory. It should perhaps be emphasized that the

test of this new diet was an extremely rigid one in that the chemically defined diet chosen for comparison purposes was one which required several years to develop employing the accepted and classical methods of nutrition. On the basis of our past experiences with chemically defined diets, there is little question that the fasting amino acid plasma profile concept will apply equally as well to the important problem for example, it should enable us to accurately and quantitatively determine just how much lysine need be added to the diet of any given species for a maximal growth response, without the necessity of carrying out the usual tedious and expensive growth studies, and additionally should enable us to determine just how much of the other essential amino acids need be added to still further increase the nutritive value of these diets. It would, of course, be of importance to ascertain whether, on the basis of this concept, we could more closely approximate the dietary needs of people suffering from protein malnutrition, from various metabolic dysfunctions (phenylpyruvic) oligophrenia, aminoaciduria, etc.), or from surgical wounds, whose amino acid requirements certainly differ from those of the normal individual. Although much remains to be done on the experimental side, there is every indication that this important concept will do much to remove nutritional studies from the realm of the empirical and place them on a sounder theoretical basis.

Proposed Course of Study: It is planned to continue the various phases of this project along the lines indicated above.

(ATTACHMENT VI.)
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Part B.

Publications other than abstracts from this project:

1. Greenstein, J. P., Otey, M. C., Birnbaum, S. M. and Winitz, M.: Quantitative nutritional studies with water-soluble, chemically defined diets. X. Formulation of a nutritionally complete liquid diet. J. Nat. Cancer Inst., 24, 211, 1960.

2. Winitz, M., Birnbaum, S. M., Sugimura, T., and Otey, M.C. Quantitative nutritional and in vivo metabolic studies with water-soluble, chemically defined diets. Jesse P. Greenstein Memorial Volume. Academic Press, New York, 1960.

3. Greenstein, J. P., Winitz, M. and Birnbaum, S. M.
   Six separate articles on:
   a. Dehydropeptidases
   b. Pancreatic carboxypeptidase
   c. Renal aminopeptidase
   d. Glutaminase
   e. Amino acid amidase
   f. Amino acid acylases
   The Biochemists Handbook, E. and F. N. Spon, Ltd. London, 1960.

4. Greenstein, J. P. and Winitz, M., Chemistry of the Amino Acids, 3 vols., John Wiley and Sons, N. Y.
   To be published, January 1961.

5. The following four manuscripts, concerned with this project, are presently in various stages of preparation:
   Quantitative nutritional studies with water-soluble chemically defined diets.
   XI. Effect of essential amino acid levels on growth.
   XII. Study of specific metabolic processes in vivo.
   XIII. Role of renal resorption in the utilization of D-amino acids.
   XIV. Use of fasting amino acid plasma levels in the formulation of nutritionally adequate diets.

Serial No. NCI-339
1. Lab. of Biochemistry
2. Physical Chemistry and Chromatography
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: The Fractionation of Oligonucleotides derived by Enzymic Degradation of Nucleic Acids.

Principal Investigators: H. A. Sober and G. W. Rushizky

Other Investigators: L. A. Heppel and M. F. Singer, Laboratory of Biochemistry and Metabolism, National Institutes of Arthritis and Metabolic Diseases.

Cooperating Units: National Institute of Arthritis and Metabolic Diseases.

Man Years (Calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: To separate and characterize nucleotide sequences derived by enzymic digestion of nucleic acids as a means of characterizing the nucleic acids or the nucleases employed.

Methods Employed: Nucleic acids are partially and completely digested by specific and non-specific nucleases and the resulting digests are fractionated by chromatography on cellulosic ion-exchangers and Sephadex as well as by two-dimensional paper electrophoresis and chromatography. The resulting fractions are examined by physical-chemical and enzymic methods.

Major Findings: It has previously been reported that all of the mono-di- and tri-nucleotides resulting from ribonuclease digests of ribonucleic acids can be separated, the separation being based primarily on size

Part B included: NO

but modified by base composition and sequence. Two-dimensional mapping techniques can also be used. Larger nucleotide sequences require modification of existing and development of new techniques. Sephadex appears to be useful but only in the presence of high concentrations of salt. Small oligonucleotides were not retarded in 0.1 M but were in 1/2 M and 1 M salt. This dependence of retardation on salt concentration may become useful as a separation method.

Fractionation studies of adenylic acid polymers, from the hexa- to the decanucleotide and larger, coupled with analytical paper chromatography, have indicated the presence of contaminating enzyme activities in the original enzyme preparation used to degrade the polyadenylic acid polymer. Homologous oligonucleotides are being used as model substances for nucleotide separations.

Significance: The inadequacy of present chemical methods for the characterization of nucleic acids and the inability of present assay methods to recognize subtle differences in the specificity of nucleases handicap the studies of the nucleic acids themselves. Separation of the products of enzymatic digestion of nucleic acids serves several purposes. They serve to characterize the enzymatic action, to characterize the nucleic acid itself in terms of its nucleotide distribution and sequence, and to obtain a variety of pure oligonucleotides which can then be used in physical-chemical and enzymatic studies.

Proposed Course of Project: These methods will be used in the study and characterization of nucleic acids of mammalian and viral origin. Consideration will be given to the possible use of the data obtained in sequence analysis. The oligo- and polynucleotides resulting from these studies will be used for physical-chemical studies (e.g. helix formation and hyperchromicity versus chain length) and for enzymatic substrate specificity and kinetic studies.

Serial No. NCI-356
1. Lab. of Biochemistry
2. Phys. Chem. and Chromatography Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: General Techniques and Concepts for the Chromatography of Macromolecules.

Principal Investigators: E. A. Peterson and H. A. Sober

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objective: To devise and prepare adsorbents for the chromatography of substances of high molecular weight; to develop criteria for testing adsorbents, such as specific adsorption capacity, efficiency in resolution, etc.; to design and test systems for continuous automatic recording of chromatographic data; to devise arrangements for the production of various gradients of pH, salt, and other variables required for the chromatography of macromolecules; to study the effect of particle size, column geometry, flow rate, temperature, etc. on the resolution obtained; and to develop and investigate fundamental concepts dealing with the processes occurring during chromatography.

Methods: Cation and anion exchangers are made from cellulose of different particle sizes by attaching ionizing groups having various acidic or basic strengths. The effects of variables under study are evaluated with respect to the resolution of test mixtures of macromolecules. Special experiments are devised for the testing of concepts.

Major Findings:
1. Two new cellulosic ion exchangers (designated ECA-cellulose and ECTHAM-cellulose, respectively) have been developed for

Part B included: YES

use in the chromatography of substances that are too tightly bound to the previously available adsorbents. Both possess basic groups that are weaker than those of DEAE-cellulose or ECTEOLA-cellulose, and they have been used successfully in the chromatography of ribosomes and soluble ribonucleoprotein -(See NCI-365).

2. New elution gradients for the chromatography of serum have been explored, and new buffer species have been tested. Tris succinate is of special interest for the chromatography of serum to be used in trace metal studies, since it is completely combustible in the carbon arc used for metal analysis. Serum albumin is eluted in much sharper peaks in the succinate gradient than when phosphate is used. Ammonium bicarbonate gradients were less effective in resolving the serum proteins than any of the other buffers studied, perhaps because the use of a decreasing pH gradient was precluded by the volatility of CO_2.

Sephadex has been successfully applied in some aspects of this work as a replacement for dialysis in buffer exchange. Its use reduces the time required by a factor of ten, a matter of some importance when unstable materials are being studied. Three grades of Sephadex have been tested for their ability to separate oligonucleotides on the basis of molecular size. Preliminary work indicates that high concentrations of salt are necessary to overcome charge effects that impede entrance of the oligonucleotides into the Sephadex granules.

3. A simpler, more convenient model of the variable gradient mixer has been developed. A device for concentrating small volumes of effluent to permit paper electrophoreses of individual chromatographic fractions (instead of pooled fractions) has been built and is being tested. Apparatus for the automatic titration of adsorbents and the monitoring of miclease reactions has been assembled and put to use.

Significance: The effectiveness of shromatography in its various applications to macromolecules depends upon appropriate adjustment to the properties of the substances under study and a correct interpretation of the results obtained. New adsorbents and elution procedures, as well as refinements in the techniques used for examining the chromatographic effluent, contribute to both.

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Serial No. NCI-356

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Proposed course of project: Development has been initiated on a system of cellulosic ion exchangers in which each exchanger will have widely separated clusters containing a uniform number of charges. Choice of the appropriate member of the series should overcome the difficulties of excessively tight binding now encountered with some nucleic acids and nucleoproteins. Such a system of ion exchangers is expected to be useful, moreover, in basic studies of the adsorption phenomenon.

Further evaluation will be made of Sephadex as a replacement for dialysis in buffer exchange and as a means of separating molecules of different size. The effect of salt concentration on the porosity of Sephadex to highly charged molecules, such as obsonucleotides, will be investigated. Studies of the displacement techniques employing mixtures of Sephadex with cellulosic adsorbents (discussed in the 1959 report) will be continued.

(ATTACHMENT III)
Serial No. NCI-356

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Peterson, E.A., and Sober, H.A.: "Diethylaminoethylcellulose", "Carboxymethylcellulose", "ECTEOLA--cellulose", and "Phosphorylated Cellulose". Biochemical Preparations 8, edited by A. Meister, John Wiley and Sons, New York, in press, 1961.

2. Peterson, E. A., and Sober, H. A.: A Variable Gradient Device for Chromatography. A Laboratory Manual of Analytical Methods of Protein Chemistry, edited by R. J. Block and P. Alexander, Pergamon Press, London, 1960, pages 88-103.

3. Sober, H. A., and Peterson, E. A.: Chromatographic Evaluation of Protein Mixtures. Greenstein Memorial Symposium, edited by J. Edsall. Academic Press, New York, 1960, pages 61-84.

4. Peterson, E. A., and Sober, H. A.: Column Chromatography of Proteins: Substituted Celluloses. Methods in Enzymology V, edited by S. P. Colowick and N. O. Kaplan, Academic Press, New York, in press 1961.

5. Peterson, E. A., and Rowland, J., A Simplified Multichamber Gradient Mixer, J. Chromatography, in press, 1960.

Numbers 1-4 were listed as "in press" in the 1959 report.

Serial No. NCI-365
1. Lab. of Biochemistry
2. Phys. Chem. and Chromatography Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Investigation of Cytoplasmic Nucleoproteins and Their Role in Protein Synthesis.

Principal Investigators: E. A. Peterson and E. L. Kuff

Other Investigators: None

Cooperating Units: None

Man Years (Calendar year 1960)
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To determine whether the ribonucleoprotein particles of rat liver and of hepatoma comprise mixtures of different species and, if so, to investigate the relationship of the individual species to the synthesis of proteins.

Methods: Cytoplasmic ribonucleoprotein particles are isolated by a combination of centrifugal and chromatographic techniques for use in metabolic experiments (to be undertaken in collaboration with Dr. R. W. Hendler of NHI) designed to follow the transfer of labeled amino acids to protein. This period has been devoted to the development of procedures and adsorbents for the chromatography of rat liver microsomes, ribosomes, and soluble RNA.

Major Findings:

1. Microsomes isolated by differential centrifugation can be adsorbed on a suitable cellulosic anion exchanger, allowing glycogen particles and residual protein to pass through unadsorbed. Microsomes low in RNA content also pass through in the initial buffer, providing a relatively simple method for their isolation from other microsomes.

Part B included: NO

2. Attempts to chromatograph the adsorbed microsomes, however, were defeated by an unusual tendency of these cytoplasmic fragments to establish additional bonds with the adsorbent, once they were immobilized on its surface, even under conditions that were marginal with respect to initial adsorption. As a result only a portion could be eluted under conditions compatible withthe preservation of their integrity. However, the adsorbed microsomes can be disrupted by passing a low concentration of a nonionic detergent through the column, washing out the lipoprotein and leaving the ribonucleoprotein particles(ribosomes)adsorbed. A gradient of NaCl subsequently elutes most of the nucleic acid-containing material as intact ribosomes. In this manner clean preparations of particles having a sedimentation constant of 77S can be obtained. A large, initial peak is followed by two smaller ones in the salt gradient, and a small additional fraction rich in ribonucleic acid is eluted by 0.1 M Na_3PO_4.

3. "Soluble" ribonucleoprotein can be obtained by passing a clear, particle free supernatant from a rat liver homogenate through a Sephadex column (for buffer exchange and removal of small molecules) and into a suitable cellulosic anion exchanger. The ribonucleoprotein is adsorbed on the latter, whereas almost everything else passes through. Elution with a low salt gradient removes a small peak of protein relatively low in absorbance at 260mμ, followed by the ribonucleoprotein. Phenol extraction of the latter by the usual procedure yielded protein and an "S-RNA". The latter, upon rechromatography behaved identically with the original ribonucleoprotein. The question of completeness of removal of protein is being studied, as well as the implications with regard to structure that arise from the identical chromatographic behavior of this RNA and the original RNP.

Significance: The cytoplasmic ribonucleoproteins, particulate and soluble, are widely recognized as a vital part of the protein synthesizing apparatus of the cell. The present work is providing means for the isolation and fractionation of these key cell fragments under milder conditions than any yet reported. The information gained and the techniques developed in this investigation are expected to aid in the study of both normal and neoplastic growth.

(ATTACHMENT II)
Serial No. NCI-3465

PHS-NIH
Individual Project Report
Calendar Year 1960

Proposed course of project:

The development of chromatographic procedures for the fractionation of cellular components of rat liver and hepatoma will be continued. Particles isolated from Novikoff hepatoma without the use of surface-active agents will be studied chromatographically. Fractions from these and other preparations will be examined to determine whether different groups of proteins are synthesized by different types of particles. A possible in vivo effect of chemical carcinogens on the ribosomes will be investigated.

Serial No. NCI-366
1. Lab. of Biochemistry
2. Phys. Chem. and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Structural and Biochemical Investigation of Intracellular Components.

Principal Investigator: Edward L. Kuff

Other Investigators: M. Potter, R. F. Zeigel, L. Law (Laboratory of Biology)

Cooperating Units: Biology Laboratory, NCI

Man Years (Calendar year 1960):
Total: 1-1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: Investigation of the cytochemical organization of normal and malignant cells, with particular reference to the problem of protein synthesis and reduplication of intracellular components.

Methods: Tissue fractionation by differential and density gradient centrifugation, analytical ultracentrifugation; enzymatic assay; electron microscopy of tissues and isolated fractions; incorporation studies of P^{32} into tissue lipids.

Results: 1. An investigation of the cytoplasmic fractions derived from mouse plasma cell neoplasms has been undertaken. The cells of these tumors have been shown by Potter and Fahey to produce and secrete into the circulating blood large quantities of myeloma proteins while simultaneously maintaining a high rate of mitosis. Electron microscopy by Dalton in this Institute has revealed the presence of an extensive endoplasmic reticulum and highly developed Golgi systems within the cells. Ribonucleoprotein RNP particles were extremely numerous, both in association with the endoplasmic reticulum and as apparently free entities in the cell sap.

Part B. included: YES

(ATTACHMENT I)
Serial No. NCI-366

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Individual Project Report
Calendar Year 1960

1a. Preliminary experiments have shown that homogenates of plasma cell tumors can be fractionated by standard methods of differential centrifugation. Furthermore, it is possible to separate the entire microsome fraction into 2 subfractions, one made up primarily of free RNP particles (such as were studied in detail in the case of the Novikoff hepatoma; see previous Annual Report, 1959) and the other composed of fragments of the endoplasmic reticulum (membranes) with associated RNP particles.

1b. Microsome fractions have been prepared from a variety of plasma cell neoplasms for use in a comparative study of mouse myeloma proteins by Dr. M. Potter. As was hoped, immunochemical methods employed by him have revealed that these microsome fractions carry material antigenically related to the circulating myeloma globulins in mice bearing the tumors. Details of this work, which has recently been submitted for publication, are presented in the Annual Report of Dr. Potter. It has been shown further that RNP particles prepared from the microsomes by deoxycholate treatment carry the antigenic groups of the myeloma proteins.

2. Virus-like particles have been shown by Dr. Dalton to occur regularly in neoplastic mouse plasma cells. He has observed that the particles were localized exclusively within the endoplasmic reticulum of these cells. Through the use of the non-ionic detergent Triton x 100, it has been possible to disrupt the membrane component of the isolated tumor microsomes in such a way as to liberate the virus-like particles in morphologically unaltered form. Testing of these preparations for biological activity is under way at present, with negative results thus far. This work is being done in collaboration with Drs. A. J. Dalton and L. W. Law.

3. The investigation of isolated Golgi material, last described in the 1958 Annual Report NCI-401, has been taken up again.

3a. Through the use of a density gradient fractionation method originally developed in this laboratory for the isolation of Golgi membranes from rat epididymus, it has been possible to isolate a similar Golgi fraction from mouse plasma cell neoplasm MCP2. The identity of the isolated material has been established with the electron microscope by Dr. R.F. Ziegel. Although material has thus far been available in limited amounts

(ATTACHMENT I)
Serial No. NCI-366

PHS-NIH
Individual Project Report
Calendar Year 1960

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3a. Through the use of a density gradient fractionation method originally developed in this laboratory for the isolation of Golgi membranes from rat epididymus, it has been possible to isolate a similar Golgi fraction from mouse plasma cell neoplasm MCP2. The identity of the isolated material has been established with the electron microscope by Dr. R.F. Ziegel. Although material has thus far been available in limited amounts

it has been shown that the tumor Golgi fractions resemble those obtained from epididymus both in their morphology and in their high content of lipids.

3b. The rate of incorporation of P^{32} into the phospholipids of isolated Golgi fractions of epididymus has been compared with the corresponding rates for other intracellular fractions. For the relatively long incorporation periods thus far studied (1-3 hrs.), there was little difference between the Golgi and other cytoplasmic fractions.

Significance for Cancer Research: 1. The work with the plasma cell tumor microsome fractions has several aspects. The use of these fractions as described in Results 1b has already provided a useful new tool in immunochemical studies of myeloma globulins in mice. The fact that myeloma proteins are associated with isolated microsomes and RNP particles indicates that the neoplastic plasma cells may be valuable in investigating the relationships between the synthesis of specific secretory proteins and that of the actual cellular proteins themselves.

2. The reasons for investigation of possible virus activity in extracts of the neoplasms are obvious in the context of present cancer research. In addition, the techniques developed may be of general usefulness in virus work.

3. The study of isolated Golgi membranes is of general significance for our understanding of cell structure and function.

Future Work: 1. The nature of the association of myeloma globulins with the microsomes of plasma cell neoplasms will be studied in more detail, particularly with regard to the possibility that the microsomes, and, more specifically, the microsomal RNP particles, may represent the sites of synthesis of these proteins. If artificial redistribution of protein during preparation of the fractions can be ruled out or eliminated, the question can then be investigated whether the myeloma globulins are associated with all of the tumor RNP particles or only with those attached to the endoplasmic reticulum. In this manner, it is hoped that an approach can be made to the question of specificity of protein synthesis among the cytoplasmic RNP elements. In addition to immunochemical and ultracentrifugal techniques already available, it is anticipated that the chromatographic methods presently being developed for the separation of ribonucleoprotein

components of liver cytoplasm (see Project Serial No. NCI-365) with Dr. E. A. Peterson) can be applied to this system.

Plasma cell neoplasm microsome fractions and extracts thereof will continue to be employed as sources of specific antigenic material for the immunochemical study of mouse myeloma globulins. This will be done in collaboration with Dr. M. Potter as principal investigator (Project Serial No. NCI-427).

2. Future work with the virus-like particles of the plasma cell neoplasms will depend upon the results of tests in progress.

3. Biochemical studies of isolated Golgi membranes will be continued, although it is anticipated that a relatively small amount of time will be spent in this work.

(ATTACHMENT IV)
Serial no NCI-366

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards and Publications

Publications:

Kuff, E. L., and Zeigel, R. F.,: Cytoplasmic ribonucleoprotein components of the Novikoff hepatoma, J. Biophysic. and Biochem. Cytol., 7: 465-478, 1960.

Honors: By invitation addressed Gordon Research Conference on Secretion, June 1960, subject "The isolation and chemistry of the Golgi complex."

Serial No. NCI-371
1. Lab. of Biochemistry
2. Phys. Chemistry and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: Studies on the nature of the D-amino acid oxidase of rat kidney.

Principal Investigator: Sanford M. Birnbaum

Other Investigators: None

Cooperating Units: None

Man Years (Calendar Year 1960)
Total: 0.5
Professional: 0.2
Other: 0.3

Project Description:

The D-amino acid oxidase activity of animal tissues (e.g. hog, sheep) has, with the possible exception of D-aspartic acid oxidation, generally been considered to be due to a single protein entity. Simple treatment of the rat kidney homogenate with acetone, however, drastically changes its relative specificity toward a series of D-amino acids. The affinity ($\frac{1}{K_s}$) of the acetone treated material for D-amino acids is less than one tenth of that of the fresh homogenate. Whether these phenomena are the result of partial differential denaturation of a mixture of oxidative enzymes or are due to some internal rearrangement of a single protein species remains to be elucidated. The latter event which appears presently to be the most probable may provide a system for the study of the intimate structure of enzymes.

Part B included: NO

Serial No. NCI-372
1. Lab. of Biochemistry
2. Phys. Chemistry and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: The Oxidation of D-amino Acids by the Rat.

Principal Investigator: Sanford M. Birnbaum

Other Investigators: M. C. Otey

Cooperating Units: None

Man Years (Calendar Year 1960):
Total: 0.8
Professional: 0.3
Other: 0.5

Project Description:

Objectives: Quantitative data on the oxidation of various D-amino acids by the intact animal are not available nor are precise measurements of the in vitro D-amino acid oxidase, levels of rat liver and kidney. Correlation of such data may give some clue as to why certain essential amino acids are utilizable by the rat when supplied as the D-isomers while others are not.

Methods Employed: Small amounts (up to 1.5 millimoles) of various D-amino acids were force fed to young albino rats in 6 divided doses over a 2-day period. The excreta were quantitatively collected and were assayed for the D-amino acid. The total excretion subtracted from the amount fed was assumed to give a measure of the in vivo oxidation.

Major Findings: The most striking observation to date is that whereas the fraction oxidized varied from about 40 to almost 100 percent, depending on the nature of the amino acid administered, this percentage value was remarkably constant for a given D-amino acid at whatever level it was fed. Study of the specific and total oxidative activity of rat kidney and liver homogenates against a series of D-amino acids has revealed: 1. that the relative specificity of the

Part B included: NO

two tissues is similar if not identical in this respect and 2. there is no apparent relation between the in vitro and in vivo oxidative rates for D-amino acids. This latter observation raises some question as to the physiological significance of in vitro enzyme measurements.

Proposed Course: This project will be extended to include a study of the effects of various biological and pathological factors such as protein starvation and tumor growth on the overall excretion of D-amino acids.

Serial No. NCI-373
1. Lab. of Biochemistry
2. Physical Chemistry and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Studies on partial enzymatic digests of ribonucleic acids.

Principal Investigator: G. W. Rushizky and H. A. Sober

Other Investigators: C. A. Knight, Virus Laboratory, University of California, Berkeley 4, California

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objectives: To study the course of the partial enzymatic hydrolysis of ribonucleic acid and to identify the sequences released.

Methods Employed: High molecular weight ribonucleic acid from tobacco mosaic virus and from yeast was digested by pancreatic ribonuclease to varying degrees. The digests were fractionated by a two-dimensional mapping procedure which combines paper electrophoresis and paper chromatography. The fractions so isolated were further examined by physical-chemical and enzymatic methods. The amounts of the compounds found in the intermediate digest were compared with the corresponding values from complete digests.

Part B included: NO

(ATTACHMENT I)
Serial No. NCI-373

PHS-NIH
Individual Project Report
Calendar Year 1960

Major Findings: As a result of comparing the amount of each compounds derived from complete and those from incomplete digests the following generalizations were obtained: a) oligonucleotides are released before the pyrimidine mononucleotides, b) with the pyrimidine mononucleotides, cytidylic acid was released before uridylic acid and c) in each case, the cyclic intermediate of cytidylic acid or its oligonucleotides were converted to the terminal 3'- form before the corresponding uridylic acid compounds.

Significance: Pancreatic ribonuclease has been found to have a preference for the enzymatic hydrolysis of pyrimidine linkages adjacent to purine linkages, i.e., these linkages are split before pyrimidine-pyrimidine bonds. The study of nucleotide sequences in soluble or high molecular weight ribonucleic acid would be greatly aided if these large polymers could be hydrolyzed to yield oligonucleotides of intermediate size which can be handled by current techniques. A preferential specificity such as just described is the first prerequisite for the preparation of large and homogeneous oligonucleotides in pure form.

Proposed Course of Project: Varying kinds of hydrolysis will be utilized in order to obtain large and different fragments that can subsequently be isolated and further degraded and characterized. Improved separation techniques remain to be worked out.

Serial No. NCI-374
1. Lab. of Biochemistry
2. Physical Chemistry and Chromatography Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Isolation and Purification of Nucleases.

Principal Investigators: G. W. Rushizky and H. A. Sober

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objectives: The recognition, purification and characterization of nucleases with hydrolytic activities directed toward specific nucleotide linkages.

Methods Employed: Conventional protein fractionation techniques including precipitation and ion exchange chromatography are utilized. The starting material is a Japanese preparation from which Takadiastase is made, and which has been reported by Egami et al (J. Biochem. (Japan) 46, 757 (1959), to contain two enzyme activities, one directed toward adenylic and the other toward guanylic acid linkages.

Results: The two enzymes, "adenylase" and "guanylase" have each been purified some 400-fold. Each preparation is free of the other activity when tested at a 1:100 enzyme :substrate ratio.

Part B. included: NO

PHS-NIH
Individual Project Report
Calendar Year 1960

Significance: These two enzymes appear to hydrolyze adenylic and guanylic acid nucleotide linkages respectively. When isolated in enzymatically pure form and after the substrate specificities have been defined, they will serve as tools for the specific cleavage of nucleotide sequences of ribonucleic acid and thus complement the pyrimidine specificity of pancreatic ribonuclease. The hydrolytic properties of these enzymes will be used in future studies of nucleic acid sequences.

Proposed Course of Project: Continued purification of these two enzymes will be sought. A search for other enzymes with different specific hydrolytic properties will be continued.

Serial No. NCI-375
1. Lab. of Biochemistry
2. Physical Chemistry and Chromatography Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Homogeneity and Structure of Proteins.

Principal Investigators: R. W. Hartley, Jr.

Other Investigators: H. A. Sober, E. A. Peterson and L. Rosenberg (General Medicine Branch, NCI).

Man Years (calendar year 1960):
Total: 2
Professional: 1 1/3
Other: 2/3

Project Description:

Objectives: To determine the nature and origins of the molecular heterogeneity observed within particular protein species as well as the presumed underlying homogeneity within such species, and the intrinsic similarities between proteins of different outward character.

Methods: Crystalline bovine plasma albumin and a number of its derivatives were fractionated by a chromatographic technique in which distinct but very similar proteins are allowed to displace one another on DEAE-cellulose. The fractions were analyzed by several physical and chemical techniques before and after treatment with a variety of reagents.

Major Findings: A high degree of resolution in the fractionation of albumin has been obtained, the most striking result

Part B included: NO

of which has been the separation of mercaptalbumin (1 mole SH/mole) and non-mercaptalbumin (no SH). Treatment of the non-mercaptalbumin with mercaptoethanol preduced about one titratable SH group per molecule and reduced its affinity for the adsorbent to that of the mercaptablumin.

Albumin dimers, fractionated from commercial material, were found to be heterogeneous chromatographically and with respect to reduction to monomer by mercaptoethanol. Dimers formed from monomers by an ethanol treatment were, by contrast, all reducible by mercaptoethanol. Rather unexpected was the observation that this ethanol treatment produced SH-sensitive dimers from purified non-mercaptalbumin in a yield equal to that for whole albumin. Blocking SH groups of whole albumin with para-chloromercuribenzoate nevertheless inhibited dimerization. A fraction assaying 95% dimer, isolated from an ethanol-treated monomer fraction, contained no titratable SH groups.

Significance: Our present results bear on the physical and chemical nature, in proteins, of the disulfide and sulfhydryl groups which are of importance in maintaining the native configuration and activity of many proteins.

Any advance in the understanding of protein structure and chemistry may shed light on the mechanisms of their synthesis and on their function, both of which are of fundamental importance in all life processes, normal and pathological. A particularly fruitful advance in this understanding would be a clarification of the nature, origins, and biological significance of the heterogeneity observed within particular protein species.

Proposed Course of Project: In view of the results reported here, the work on plasma albumin fractions will continue. The anomolous behavior of the non-SH albumin will be investigated further. Experiments using C^{14} labelled iodoacetate as an SH marker are in progress with the aim of determining whether there is heterogeneity with respect to the

location of the free SH on the peptide chain, and whether its location can be changed by intermolecular sulfhydryl-disulfide exchange. This label will also be used in examining the mechanisms of dimer and polymer formation. Further attention will also be given to the monomeric heterogeneity resulting from factors apparently unrelated to the sulfhydryl titer, and to the nature of the stable dimer component.

Serial No. NCI-376
1. Lab. of Biochemistry
2. Physical Chemistry and Chromatography Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: The Utility of Water-soluble Chemically Defined Diets for Rats with Intestinal Occlusion.

Principle Investigator: Miles C. Otey

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 0.5
Professional: 0.3
Other: 0.2

Project Description:

Objectives: The primary objectives of the project are to investigate the possible advantage of employing water-soluble diets for the maintenance of rats with intestinal obstruction as compared with the feeding of a conventional diet of natural foods, and to assess the therapeutic value of the use of water-soluble diets in combination with tumor-inhibitory agents for the control of malignancies of the lower digestive tract.

The project was initiated recently and is still in its preliminary stage. However the production of intestinal obstruction will be investigated (a) by implanting tissue from various tumors in the intestinal wall, (b) by inducing intestinal tumors with chemical carcinogens, and (c) by mechanical means.

Part B included: NO

(ATTACHMENT I)
Serial NO. NCI-376

PHS-NIH
Individual Project Report
Calendar Year 1960

Water-soluble diets and conventional control diets will be fed to animals with established occlusions, and the average survival times and the well-being of the animals on the different regimens will be noted. Similar experiments will be carried out concomitant with anti-tumor treatment employing agents of established worth.

Significance: The significance of the potential findings to biomedical research are obvious. Various pathological conditions that involve the lower portions of the alimentary canal might respond more favorably to treatment in conjunction with collateral use of diets of this type.

Serial No. NCI-3707
1. Lab. of Biochem.
2. Phys. Chem. and Chromatography Section

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Studies on Cyclic Polypeptide Antibiotics

Principal Investigators: N. Izumiya and M. Winitz

Other Investigators: None

Cooperating Units: Dr. A. Saz of NIAID

Man Years (Calendar Year 1960)
Total: 2.5
Professional: 1.5
Other: 1.0

Project Description:

Polypeptide antibiotics such as gramicidin S and tyrocidin A possess in common several structural features, namely, (a) a basic character due to the presence of one or more diamino acid residues, (b) a cyclic conformation, and (c) at least one amino acid residue of the D-configuration. The simplest cyclic polypeptides which exhibit these structural features are 2:5-diketopiperazines wherein one or both of the component amino acid residues possess a D-configuration and a basic character. The four stereomers of valyllysine anhydride were synthesized as model compounds and examined for possible antibacterial activity.

Synthesis was achieved by the carbodiimide-induced condensation of formyl-L(or D)-valine with ε-carbobenzoxy-L(or D)-lysine methyl ester, followed by treatment of the resulting condensation product with methanolic-HCl to remove the formyl blocking group. The dipeptide ester hydrochlorides so derived were converted by the action of methanolic-NH_3 to the corresponding carbobenzoxylated anhydrides and these, in turn, transformed to the desired unacylated valyllysine anhydrides (isolated as the hydrochloride salts) by palladium-catalyzed hydrogenolysis. In addition, each of the four stereomers of valyllysine was synthesized by condensation via mixed anhydrides of carbobenzoxy-L(or D)-valine with ε-carbobenzoxy-L(or D)-lysine methyl ester followed by successive saponification and hydrogenolysis of the acylated dipeptide esters so derived.

Part B included: NO

PHS-NIH
Individual Project Report
Calendar Year 1960

The four stereomers of phenylalanyllysine anhydride were similarly prepared.

The valyllysine anhydrides and the phenylalanyllysine anhydrides proved insusceptible to the hydrolytic action of trypsin and chymotrypsin. None of the stereomers of either compound exhibitied antibacterial activity toward either gram positive or gram negative bacteria.

Significance: The study indicated that another factor or factors aside from a basic character, the incorporation of a D-amino acid residue and the possession of a cyclic conformation are necessary for a polypeptide to reveal antibiotic activity. The additional feature required could very well be that of molecular size, and it was with this in mind that the synthesis of the cyclic hexapeptide, cyclo-D-valyl-L-phenylalanyl-L-lysyl-D-valyl-L-phenylalanyl-L-lysyl, was undertaken. Synthesis of this compound is not yet complete.

Proposed course of investigation: The project was terminated with the decision of Dr. Izumiya to transfer to another Laboratory.

Serial No. NCI-330
1. Lab. of Biochemistry
2. Cytochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Combined Actions of Anti-Cancer Agents and Hormonal Systems that Inhibit Cancer Metabolism and Growth.

Principal Investigators: D. Burk, M. Woods and K. Wight

Other Investigators: J. Hunter, B. Stambuk (Research Fellow), J. F. Seitz, (World Health Organization) Visiting Scientist, and T. Howard.

Cooperating Units: E. P. Vollmer, Endocrinology Section Cancer Chemotherapy National Service Center; M. Landy and M. Shear, Laboratory of Chemical Pharmacology, NCI.

Man Years (Calendar Year 1960):
Total: 2
Professional: 1-1/2
Other: 1/2

Project Description:

Objectives: To determine the action and mode of action of anti-cancer agents when used in vitro and in vivo in conjuction with relatively tumor-specific inhibitors of glucose utilization, and/or other metabolic processes.

Methods: Tumor materials are either exposed in vitro to the compound (s), or compound(s) is (are) administered in vivo, and the effects on invitro metabolism subsequently determined. Methods of metabolic analysis developed in connection with studies of insulin:anti-insulin hormonal metabolic regulatory systems are employed. A series of tumors exhibiting a spectrum of hormonal sensitivities are employed as test systems. Effects of treatments on tumor growth and viability are measured with appropriate techniques.

Major Findings: During 1960 studies of the action of fluorinated pyrimidines, and related compounds, on tumor metabolism and growth were continued. In collaboration with

Part B included: YES

(ATTACHMENT I)
Serial No. NCI-330

PHS-NIH
Individual Project Report
Calendar Year 1960

Dr. Joseph F. Seitz (Visiting Scientist from the Institute for Hematology and Blood Transfusion, Leningrad, U.S.S.R) the effects of fluorinated pyrimidines on "high energy phosphate" metabolism of tumor cells were studied. The results showed that at low levels of inorganic phosphate, where in vitro glycolytic inhibition may reach 80%, there was no reduction in net acid-insoluble high energy phosphate. All of this phosphate fraction, however, may not have consisted of simply ADP and ATP, but might have involved possible fluorinated derivatives.

The glucose requirements for anti-tumor action (glycolytic inhibition, growth inhibition) have been investigated further. While inhibitory action by a series of 5-fluorinated pyrimidines seems to depend on the presence of an active glucose metabolism during exposure to the drug, in a few instances, following in vivo exposure to 5-fluorouracil, the transplantability of ascites tumor cells was markedly inhibited without concomitant glycolytic inhibition. However, in all instances, there was a strong positive correlation between the in vitro glycolytic inhibitory activities of a series of substituted pyrimidines and their in vivo anti-tumor activities.

Evidence was obtained that the anti-glycolytic pyrimidines inhibit aerobic glycolysis in ascites tumor cells by competitively inhibiting the utilization of inorganic phosphate during initial stages of exposure to the drug. A secondary phase of inhibition is not reversed by increasing the concentration of inorganic phosphate in the medium.

A recent report by Lemon (Cancer Chemotherapy Reports 8:97-101, July, 1960) indicates that simultaneous intravenous administration of glucose with 5-fluorouracil in man is accompanied by reduction in host toxicity, with retnetion of anti-cancer effects.

Results obtained in this project point to pyrimidines (natural and 5-fluorinated) as useful tools for investigating the mechanisms involved in oxidative phosphorylation. Cytoxan, a cyclic phosphoramide derivative of nitrogen mustard, was found to inhibit tumor cell glycolysis. Comparison of the L1210 ascites strain resistant to cytoxan, developed by Dr. Lane, with the L1210 ascites susceptible strain, showed a difference in the glycolytic and respiratory properties of

the two strains with respect to cytoxan action. Cytoxan inhibited the glycolysis and respiration of the susceptible strain considerably more than in the resistant strain, the glycolysis and respiration of the tumor being determined after action of cytoxan in vivo. Nicotinamide and diphosphopyridine nucleotide reversed cytoxan action in the resistant more than in the susceptible strain. Further progress on the activation of cytoxan in vitro was made. In the presence of triphosphopyridine nucleotide (TPN) and magnesium, as well as adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN), cytoxan inhibition of glycolysis of liver and mammary tumor homogenates and derived subcellular elements was obtained. Thus a connection of cytoxan action with magnesium and TPN was found. An inhibition of ATP-ase by 5-fluorouracil was demonstrated for the L1210 ascites tumor cells.

Further study of the respiration and glycolysis of tumors and normal tissues was undertaken. Glucose usually produced a pronounced Crabtree effect (inhibition of respiration by glucose) in the case of ascites tumors, but this was not always true. TPN, with increased magnesium and nicotinamide, increased the glycolytic production of carbon dioxide three to four times above the amount produced in the presence of ATP and DPN only, in mouse mammary tumor homogenates, but not in liver homogenates. An increase in the amount of lactic acid produced was also found. This finding has been extended to whole cells for the thymoma ascites. The increased glycolysis in the presence of TPN is not so great with cells, but clearly demonstrable. There is no evidence to support the hypothesis that DPN is formed from TPN. Other nucleosides such as inosine, uridine, cytosine and guanosine triphosphates could be substituted for adenosine triphosphate partly or wholly in tumor glycolysis, in studies with thymoma ascites and mammary tumor homogenate. These studies are being extended.

In collaboration with Drs. Landy and Shear of the Laboratory of Chemical Pharmacology, normal blood serum from many animal species was found to exert in vitro lethal effects on cells of various mouse tumors as measured by cytologic changes and abolition of glycolysis. The system in serum responsible for the cytocidal effect consisted of a natural antibody and complement. This antibody had specificity for an antigen present in tumors and lymphoid tissue of mice. The effects of toxicity of the external medium, magnesium and/or calcium requirements and kinetics of cell destruction at various

incubation temperatures were determined. In collaboration with Dr. Robert Smith, Department of Surgery, NCI, the levels of cytocidal activity (mouse ascites tumor test system) in the serum of cancer patients were compared with normal human serum. The action of such sera (active and 52°C.- inactivated) on the patients own cancer cells was also investigated. These results, though only preliminary, indicate somewhat higher than normal levels (2 to 3-fold) of cytocidal activity in the sera of certain cancer patients.

Proposed Course of Project: Further tests of sera from cancer patients are planned in conjunction with chemotherapeutic studies.

(ATTACHMENT IV)
Serial No. NCI-330

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B: Honors, Awards and Publications

Publication other than abstracts from this project:

1. Woods, M., Hunter, J. and Burk, D. Glucose enhancement of inhibition by fluorinated and normal pyrimidines of tumor glycolysis and transplantability. Proc. Amer. Assoc. Cancer Res., 3: 163, March 1960.

2. Hunter, J., and Woods, M. Effects of 5-fluorouracil and 5-fluorodeoxyuridine on aerobic glycolysis in rat bone marrow. Proc. Amer. Assoc. Cancer Res. 3:122, March 1960.

3. Wight, K., Burk, D., Woods, M. and Lane, M. Inhibitory action of cytoxan in vitro and in vivo on tumor respiration and glycolysis. Proc. Amer. Assoc. for Cancer Res. 3: 162, March 1960.

4. Landy, M., Michael, J., Trapani, R.J., Achinstein, B., Woods, M. and Shear, M. J. A complement-dependent system of normal serum lethal to mouse tumor cells. Fed. Proceed. 19: 394, March 1960.

5. Woods, M., Landy, M., Michael, J., Trapani, R. J., Achinstein, B., and Shear, M. J. Abolition of glycolysis in mouse tumor cells by a complement-dependent cytotoxic system of normal serum. Fed. Proceed. 19: 139, March 1960.

6. Landy, M., Michael, J., Trapani, R. J., Achinstein, B., Woods, M. and Shear, M. J. Cancer Research 20: 1279-1291, Oct. 1960.

7. Stambuk, B. and Burk, D. Cytotoxic action in vitro of Human Serum on the Metabolism of Mouse Ascites Cells, and Counteraction by Endotoxic and other Polysaccharides. J. Nat. Cancer Inst. (In press)

8. Stambuk, B. Human Bone Marrow (book review) J. Histochem. and Cytochem. (In press)

Serial No. NCI-331
1. Lab. of Biochemistry
2. Cytochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Subcellular localization of Enzymes involved in the Carbohydrate Metabolism and Growth of Tumor.

Principal Investigators: Mark Woods, Dean Burk and Karl Giger (Research Fellow).

Other Investigators: None

Cooperating Units: M. Landy and James Whitby (Laboratory of Chemical Pharmacology).

Man Years (Calendar Year 1960)
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: This project was largely inactive during 1959. It was fully activated with the arrival of Dr. Karl Giger (Research Fellow) in October 1960, who is extending previous investigations dealing with the sub-cellular control of glycolysis by insulin and anti:insulim hormones. He will also investigate the action of bacterial endotoxic lipo-polysaccharides at the subcellular level, especially in relation to their demonstrated insulin-like action at the cellular level in vitro and in vivo.

Significance: An understanding of the precise mechanism of drug action against tumor cells requires a knowledge of the localization of the affected enzymes within the cell. Because of the insulin-like stimulation of cellular glycolysis by endotoxic polysaccharides, and the many suggested relationships of their effect to in vivo actions of endotoxic polysaccharides (on both normal and tumor cells), it is imperative to determine whether or not endotoxin action occurs at the mitochondrial level. If typical endotoxin action clearly

Part B included: NO

occurs at a subcellular level then current ideas concerning its presumed action at the outer cell surface might have to be extensively modified. More generally the possible etiologic role of aberrant mitochondria, or other hereditary particulates of the cytoplasm, must be considered in relation to the enzymic properties of such structures.

Serial No. NCI-332
1. Lab. of Biochemistry
2. Cytochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: Effects of Steroid Hormone Balance on Growth of Transplanted Mouse Tumors.

Principal Investigators: J. C. Hunter

Other Investigators: M.W. Woods and D. Burk

Cooperating Units: Endocrinology Section, CCNSC

Man Years (Calendar Year 1960):
Total: 1/3
Professional: 1/3
Other: None

Project Description:

Objectives: To determine the effects of manipulation of host hormonal balance on growth of transplanted mouse tumors.

Methods: Tumors are available which are sensitive to stress-modifiable hormonal mechanisms (insulin: anti-insulin system) which affect tumor metabolism and growth via regulation of the hexokinase reaction.

While the same hormonal systems strongly regulate the metabolism of normal tissues over the same mechanisms, glycolytic homeostasis is maintained to an extent not possible in tumors. Effects of manipulation of host endocrine balance by physical, chemical, or biologic means are measured in terms of tumor growth rate.

Major Findings: No new major findings this year.

Significance: These studies help to furnish a basis for the development of combination therapies in which steroid components are involved.

Part B included: YES

(ATTACHMENT I)
Serial No. NCI-332

PHS-NIH
Individual Project Report
Calendar Year 1960

Proposed Course of Project: It is planned to continue the present general approach using additional steroids and other types of chemotherapeutic agents.

(ATTACHMENT II)
Serial No. NCI-332

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B: Honors, Awards and Publications

Publication other than abstracts from this project:

1. Hunter, J. C. and Woods, M.W., Effects of 5-fluorouracil and 5-fluorodeoxyuridine on aerobic glycolysis in rat bone marrow. Proc. Amer. Assoc. for Cancer Res. 3: 122, 1960.

2. Woods, M. W., Hunter, J.C., and Burk, D. Glucose enhancement of Inhibition of Fluorinated and Normal Pyrimidines of Tumor Glycolysis and Transplantability. Proc. Amer. Assoc. for Cancer Res. 3: 163, 1960.

Serial No. NCI-333
1. Lab. of Biochemistry
2. Cytochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Fundamental Oxidative and Glycolytic Metabolism in Tumors and Tissue Cultures.

Principal Investigators: D. Burk and J. Hunter

Other Investigators: R. Madden, K. Wight and M. Woods

Cooperating Units: V. P. Perry, Tissue Bank, Naval Medical School, National Naval Medical Center

Man Years (calendar year 1960):
Total: 1-1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: The purpose of these studies is to define the effect of antimetabolites and other chemotherapeutic agents on the metabolism of cells obtained from patients with the various forms of leukemia and other cell types. New emphasis has been placed on defining the nature of anti-tumor effects of thermophilic algae observed in vitro and in vivo.

Methods: 1) Cellular preparations were obtained from patients who had a great predominance of blast cells and abnormal lymphcytes in their peripheral blood. These were separated from red cells by means of the fibrinogen sedimentation technique. Glycolysis and oxygen uptake of cells were measured in Warburg manometers in the presence of serum, glucose, bicarbonate, and the drug under analysis. Excellent single-cell preparations from several solid human and mouse tumors were

Part B included: YES

prepared by stirring scissored tumor sections in media containing the enzymes trypsin and DNA-ase, centrifuging the strained supernatant and taking the precipitated cells up in medium without trypsin.

(2) Thermophilic Chlorella (strain 7-11-05) were grown in an atmosphere of 5% CO_2 in air using a standard growth medium in Drechsel gas washing bottles. Algae thus cultured were inoculated intraperitoneally in mice and their ability to use glucose was observed as a function of the hypoglycemia induced. The Warburg manometric technique was employed to determine the glucose utilization by algae in vitro. Glucose concentration was determined chemically using the Glucostat (glucose oxidase) method in both in vitro and in vivo studies.

Major Findings: Single-cell preparations from solid tumors were compared metabolically and for viability (quantitative in vivo mouse inoculation studies) with single-cell preparations of the same tumor strains grown in ascites form. The single cells from the solid tumors behaved essentially the same as the ascites single cells. The results offer the hope of greatly facilitating metabolic studies of solid human tumors and also of obtaining single-cell-cloned tissue cultures of a variety of normal and malignant cells from human and animal tumors. Work is now in progress in cooperation with the tissue culture laboratory of the National Naval Medical Center to obtain cine-photomicrograph records of prepared single cells in culture using time-lapse photography. It is hoped that such cell motion studies will aid in establishing the viability of individually identified single cells prepared from solid tumors.

A strain of thermophilic chlorella (Sorokin and Myers 7-11-05) with an optimum temperature of 39°C. caused marked inhibition of anaerobic and aerobic glycolysis of Ehrlich and Krebs-2 mouse ascites cells when mixed in approximately equal volumes of cells in Krebs-Ringer medium. The algae consumed glucose at a rate several times faster than the cancer cells (on a unit cell volume basis). The algae, like the tumor cells, can produce lactic acid from glucose, but D-lactic acid instead of L-lactic acid is formed. When thermophilic

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Chlorella cells are introduced into the peritoneal cavity of mice, severe and chronic hypoglycemia occurs eventually resulting in death of the animal. Severe hypoglycemia (blood sugar circa 55mg %) has been measured four hours after intraperitoneal injection of 70-100 cmm. algae in 1 ml. of physiological saline. Simultaneous injection of the proper amount of dextrose with the algae p rotects against the hypoglycemic effect. Blood sugar levels of 25 mg. % have been measured 17 hours after inoculation of 20 cmm. of algae. Cells washed from the peritoneal cavity of a mouse, where they had remained for 17 hours, grew vigorously when placed in the proper medium, atmosphere, and light. Animals with hypoglycemia incurred by a prolonged exposure to 20 cmm. of algae fleetingly returned to a normal blood sugar level following intraperitoneal injection of 20 mg. dextrose, but after 2 hours they were again as hypoglycemic as before injection of glucose. Heating the algae at 80-85° C. for 15 minutes destroyed the capacity of these cells to cause hypoglycemia in mice and to utilize dextrose in vitro. Heating for 20 minutes at 60-65° C. or freezing and thawing 3 times using an ethanol/CO_2 ice bath reduced but did not totally eliminate the ability of the algae to utilize glucose in vitro. Algae heated at 60-65° C. for 20 minutes caused a less severe hypoglycemia in mice than did untreated cells after 17 hours incubation in mice.

Significance: These studies are aimed at ascertaining and understanding metabolic peculiarities of malignant cells, under conditions approaching in variable degree the in vivo state, and under conditions of chemotherapeutic action, with added attempts to discover new agents of the latter type by looking first, not last, for metabolic indication of potential chemotherapeutic action as is well illustrated by the striking me tabolic effects on tumor mechanism of which effects yet remains to be worked out.

The newer studies have revealed a most interesting phenomenon - the ability of an aquatic, unicellular green plant to live parasitically in the peritoneal cavity of a rodent by utilization of the glucose stores of the animal in a manner similar to that employed by the ascites cancers. The proper application of these algae can provide a practical means of inducing a glucose-reversible hypoglycemia by non-hormonal means, superficially resembling the end result of in vivo insulin action.

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Proposed Course of Action: It is planned to continue the present general approach. Studies will be carried on by classical research methods, by proceeding from one finding to the next, in an attempt to further delineate fundamental oxidative and glycolytic metabolic mechanisms in tumors, tissue cultures and even normal tissues.

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Part B: Honors, Awards and Publications

Publications other than abstracts from this project:

1. Burk, D., Wight, K. and Howard, T.: Inhibition of tumor cell glycolysis and growth by thermophilic Chlorella. Proc. Amer. Assoc. Cancer Res. 3: 99, 1960.

2. Wight, K., Burk, D., Woods, M. and Lane, M.: Inhibitory action of cytoxan in vitro and in vivo on tumor respiration and glycolysis. Proc. Amer. Assoc. for Cancer Res. 3: 162, 1960.

Serial No. NCI-334
1. Lab. of Biochemistry
2. Cytochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: The occurrence and function of a stress-modifiable anti-insulin hormonal complex regulating glucose utilization in cancer and normal cells.

Principal Investigators: Mark Woods and Dean Burk

Other Investigators: B. Stambuk (Research Fellow), K. Giger (Research Fellow), J. Hunter and T. Howard.

Cooperating Units: M. Landy and J. Whitby (Research Fellow) Lab. of Chemical Pharmacology, NCI and E. P. Vollmer, Endocrinology Section, Cancer Chemotherapy National Service Center.

Man Years (calendar year 1960):
Total: 1-1/3
Professional: 1
Other: 1/3

Project Description:

Objectives: To determine the extent to which insulin:anti-insulin hormonal regulation of glycolysis functions as a metabolic control mechanism in cancerous and normal mammalian cells, and to determine ways in which this mechanism can be manipulated as an aid to in vivo therapy and diagnosis.

Methods: The responses of tumor or normal tissues in vitro and in vivo to specific anti-insulin inhibitors of glycolysis, and to substances which counteract this inhibition (e.g. insulin, endotoxic polysaccharides), are determined. Tumors presenting a known spectrum of sensitivities to anti-insulin-insulin hormonal regulation, as well as clinical materials

Part B included: YES

of unknown sensitivity, are employed in these studies.

Major Findings: Human normal and leukemic leucocytes have been studied with special reference to their glycolytic responses to anti-insulin steroids, insulin and endotoxic lipopolysaccharides. Results obtained to date, though not conclusive, indicate that glycolysis in lymphocytic cells (normal and leukemic) is much more sensitive to inhibition by steroids than is the case with cells of the myelocytic series. A point of particular importance is whether more acute forms of leukemia differ in their insulin:anti-insulin sensitivity from less acute forms of normal cells. Dats suggest that acute forms may be more steroid resistant, but too few cases have been studied to be certain. The data indicate that insulin:anti-insulin regulation of glucose metabolism plays an important role in the metabolism of leukocytes (lymphocytic and myelocytic), and that endotoxic polysaccharides counteract such glycolytic inhibition in a manner resembling, although not identical with, insulin. In collaboration with Dr. James Whitby we have found similar effects of endotoxin and steroids on mouse macrophages.

A series of endotoxic polysaccharide preparations and products have been studied on the one hand with respect to their in vitro glycolysis-stimulating power, and on the other with respect to their in vivo properties (Dr. Landy and Associates). The data show a remarkably close correlation between the two types of action. In vivo administration of sub-lethal doses of endotoxin cause long-persistent elevations of glycolysis (measured subsequently in vitro) in mouse spleen and macrophages.

Significance: The data have an important bearing on the mechanism of action of endotoxic polysaccharides on mammalian cells. The stress-modifieable insulin:anti-insulin system appears to play an important role in the metabolic regulation of leukocytes, and this system appears to be involved in the action of bacterial lipopoly-saccharides on these cells.

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Proposed Course of Project: Particular attention will be given to the metabolic basis for sequential therapy with two or more agents. In cooperation with Dr. Landy and associates the action of bacterial endotoxins on the macrophages of mice will be studied with respect to both their glycolytic and disease-resistance actions. The action of endotoxins at subcellular levels in tumor cells will be investigated with Dr. Karl Giger.

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Part B. Honors, Awards and Publications

Publications other than abstracts from this project:

1. Woods, M. W. The metabolic action of endotoxins, In preparation for Soc. of Amer. Bacteriologists Symposium on Endotoxic polysaccharides to be held in Chicago, Ill. in April 1961).

Serial No. NCI-320
1. Lab. of Biochemistry
2. Enzymes and Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Studies on the rates and kinetics of enzyme synthesis and destruction in vivo.

Principal Investigators: Miloslav Rechcigl, Jr. and Vincent E. Price

Other Investigators: Robert W. Hartley, Physical Chemistry and Chromatography Section, NCI
Seymour H. Wollman, Laboratory of Physiology National Cancer Institute

Cooperating Units: None

Man years (calendar year 1960):
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: To develop simple techniques for measuring the rates and kinetics of enzyme synthesis and destruction in vivo. To study how the rates are altered in starvation and on a protein-free regimen and to compare these states with those seen in hypophysectomy and in the tumor-bearing host.

Methods Employed: The method for determining the kinetics of synthesis and destruction of the enzyme catalase in vivo by use of 3-amino-1,2,4-triazole was described in last year's report (NCI-320, 1959).

In order to check the validity of the above method, an alternative technique was used in which catalase synthesis was blocked by

Part B included: YES

use of allylisopropylacetamide, 200 mg. per kg., intraperitoneally twice daily.

When catalase synthesis was blocked, activity of the remaining catalase disappeared with a first order constant, K_D, of 0.023, corresponding to a rate of disappearance of 2.3% per hour. The first order constant for catalase destruction obtained by the use of allylisopropylacetamide was almost identical to that obtained by use of aminotriazole, thus demonstrating that the rate constants obtained by the two techniques were not significantly altered by the drugs being used.

By substituting, K_D, in the equation $K_S = K_D C_N$, where C_N is the catalase activity of the normal plateau, the rate of catalase synthesis, K_S, can readily be calculated.

Major Findings: Initial studies on the effects of starvation on catalase synthesis were presented in last year's report. Subsequent studies have verified these findings and shown that in the liver of starved animals synthesis and destruction proceeded at essentially the same rate per gram of tissue as in normal animals for the first 5 or 6 days of starvation. The liver is continually decreasing in size during this period, however, so that there is actually less catalase being synthesized and destroyed in the total liver. Since it is known that there is marked loss of the liver RNA during starvation, it is suggested that in the early stages of starvation the animal may control the rate of catalase synthesis by progressively decreasing the number of synthesizing units in proportion to the liver size, but that those synthesizing units which remain operate at full activity.

In contrast to the picture seen in starvation, animals on a protein-free diet of carbohydrate, fat, minerals, vitamins, and a source of fiber had a decreased rate of catalase synthesis per gram of tissue, or per mgm. of N. Although there was less catalase being synthesized, the fraction of catalase molecules being destroyed per unit time was within normal limits, thus resulting in a lower concentration of catalase within the liver. However, on the protein-free diet there was only a small

decrease in liver size and in the total liver nitrogen, in contrast to starvation where a marked decrease in both liver size and total nitrogen was observed.

Starvation is often referred to as a pseudohypophysectomy, in that the two states have much in common. Studies on the liver catalase of hypophysectomized animals revealed a similar picture to that seen in starvation with livers only 1/3 the normal size and with the level of liver catalase per gram at normal or slightly elevated levels.

Recently Utsügi (Gann 51: 17-25, 1960) has reported that hypophysectomy will prevent the depression of liver catalase activity of tumor-bearing animals. In our laboratory, however, when Wollman thyroid tumors were implanted subcutaneously into animals which had been hypophysectomized two months previously, a marked fall in the concentration of liver catalase was seen so that two weeks after transplantation the catalase activity was 40% of that observed in the hypophysectomized controls. This experiment showed that the effects of hypophysectomy and tumor-growth are additive, and that hypophysectomy does not block the effect of the tumor on the liver catalase.

The effect of tumors is more nearly duplicated by the protein-free diet, for in the tumor-bearing animals there is a marked fall in the catalase activity per gram liver, and the liver is frequently somewhat enlarged. In both of these states the total liver catalase is markedly reduced as it is in starvation, but it is not accompanied by the marked decrease in liver size or liver nitrogen seen in starvation.

The marked loss in liver size and total nitrogen seen in starvation has been shown by a number of investigators to result from a shrinkage in the size of the liver cells rather than from a decrease in the number of cells present. Therefore both in starvation and on a protein-free diet there is marked reduction in the catalase activity per liver cell, but on a protein-free diet there is little loss in the size or nitrogen content of the cell as compared to the marked loss seen in starvation.

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Significance to Cancer Research: The data presented above indicate that the effect of a tumor on the liver catalase is not the result of a deficiency in the energy required for synthetic processes, since if this were true one might expect to see the marked shrinkage in liver size and loss of liver nitrogen which is observed in starvation. In as much as the picture in the tumor-bearing animal is simulated by a protein-free diet, it is possible that the effect of a tumor is to produce a deficiency in one or more of the essential amino acids required for protein synthesis. The lowering of liver catalase by hemorrhage as reported by Greenfield and Price (Acta Unio Intern. contra Cancrum 15: 888-892, 1959) could also be mediated by an amino acid deficiency, since Whipple ("Hemoglobin, Plasma Protein, and Cell Protein", C. C. Thomas, Springfield, Ill.) have shown that hemoglobin takes priority over most other proteins in their demands on the supply of available amino acids. It should be kept clearly in mind, however, that a deficiency of other essential nutrients could also produce a similar picture to that observed on a protein-free diet or in the tumor-bearing animal.

Proposed Course of Research: Since the above data indicate that a deficiency in some essential nutrient other than carbohydrate or fat is a likely cause of the depression of liver catalase in the tumor-bearing animal, an attempt will be made to find the relative effects of the various essential nutrients on the tumor-bearing host.

If some nutrient essential for protein synthesis is deficient, why do not the other proteins of the liver cell fall at a rate comparable to that of liver catalase? The answer to this question appears to lie in the relative rates of protein destruction and turnover within the cell. Apparently certain structural proteins are not destroyed as long as there is sufficient energy available to maintain them in their highly structured state. If the energy source is absent, as in starvation, then these proteins too are progressively broken down and their amino acids reutilized, thus prolonging the life of the animal until a new source of food can be found. An elucidation of these processes will be of increasing importance to us in our future work.

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Part B. Honors, Awards and Publications.
Publications other than abstracts from this project:

1. Price, V. E., Rechcigl, M., Jr., and Hartley, R. W., Jr.: Methods for determining the rates of catalase synthesis and destruction in vivo. Nature, in press.

Serial No. NCI-353
1. Lab. of Biochemistry
2. Enzymes and Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: The Chromatographic Analysis of the Ribonuclease Activities in Normal and Neoplastic Tissues.

Principal Investigator: Mary E. Maver

Other Investigators: Antoinette E. Greco and George Rushizky

Man Years (calendar year 1960)
Total: 1-1/2
Professional: 1/2
Other: 1

Project Description:

Objectives: (1) To separate, by ion exchange chromatography, the multiple RNase activities of normal and neoplastic tissues. (2) To characterize these activities.

Methods employed: The tissues being analyzed are beef spleen and liver, rat liver, two transplantable rat hepatomas and a rat lymphosarcoma. Since other investigators have reported RNase activities with characteristics different from those we have found, there is the possibility that the chemical methods of separation of the activities have altered the RNase proteins. Therefore, crude extracts of liver and spleen containing those proteins soluble and those insoluble at pH 4.5 are analyzed chromatographically on DEAE. Nuclease preparations with high RNase activity were also subjected to chromatography on DEAE and the peaks of RNase activity are being rechromatographed on CM. The RNase activities are characterized by 1) their pH optima on highly polymerized rat liver RNA, 2) the products isolated from their hydrolysates when cyclic purine and pyrimidine nucleotides are used as substrates.

Part B included: NO

3) the products of hydrolysis isolated from hydrolysates of yeast RNA by paper electrophoresis followed by paper chromatography, 4) the products of their hydrolyses of polyadenylic and polycytidylic acids, 5) the effects of Mg ions and heat upon their RNase activities.

Major findings: Two enzymatic activities which fractionate closely with the RNase activities have been isolated by repeated fractionation on CM columns. They are 1) an acid phosphatase which preferentially dephosphorylates the 3'-adenylic acid, and 2) a phosphodiesterase, without RNase activity, which hydrolyzes cyclic adenylic acid to yield the 2'-adenylic acid. The latter activity had been used to characterize the acid RNase of spleen. These enzymes are much more active in calf spleen and liver and rat hepatoma 3683 than in normal rat liver.
Calf Spleen RNases. - An active "acid" RNase with a pH optimum of 5.7 on RNA has been isolated which does not hydrolyze cyclic adenylic acid but does hydrolyze cyclic cyticylic acid, and polyadenylic and polycytidylic acids to yield the 3'-derivatives. With yeast or rat liver RNA as substrate both purine and pyrimidine linkages are broken and no core remains. The alkaline RNase of spleen hydrolyzes cyclic adenylic and cytidylic acids to give the 3' acids. This RNase requires Mg^{++} ions for maximum activity and this resembles the pancreatic RNase.
Rat Liver RNases. - Rat liver was found to have an RNase with a pH optimum of 6.5-6.7 besides the activities with optima of pH 5.7 and 7.3-7.8. The 6.5-6.7 RNase is masked in crude preparations by an inhibitor which can be removed by heat or by p-chloromercuribenzoate. The RNases of liver are considerably less active than the RNases of the other tissues studied.

Significance: The RNase activities promise to be one of the main tools by which the nucleotide sequences of virus or tissue RNA can be determined, and by which the contribution of specific nucleotide linkages to the metabolism of the organism can be elucidated.

As the role of ribonucleic acid in protein synthesis becomes more evident, it becomes increasingly important to learn how the RNases function in the control of protein synthesis. Isolation of the enzymes involved and identification of the chemical bonds hydrolyzed provide an excellent basis on which to begin such a study.

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Proposed course: It is planned to identify further the mono, di and tri nucleotides which are products of the hydrolysis of RNA by these enzymes. Further chromatographic fractionation of the rat lymphosarcoma and the hepatoma N nuclease preparations will soon be undertaken.

Serial No. NCI-359
1. Lab. of Biochemistry
2. Enzymes and Metabolism Sect.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Studies on the fractionation of erythrocytes and cells of other tissues in normal and tumor-bearing animals.

Principle Investigator: Robert E. Greenfield

Other Investigators: Betty Whitaker

Cooperating Units: Radiation Therapy Service, Radiation Branch, NCI

Man Years (calendar year 1960):
Total: 2-1/2
Professional: 1
Other: 1-1/2

Project Description:

Objectives:
Part 1: In previous studies (J. B. C. 220, 607-618, 1956) on the isolation of cell particulates, it was noted that the addition of the high molecular weight polymer, polyvinylpyrrolidone (PVP), to solutions of sucrose resulted in a marked protection of the cellular particulates. It was also found that it was much more difficult to disrupt the liver cells in PVP-sucrose solutions, so that the nuclear fractions were always contaminated with large numbers of intact cells. In view of the failure of previous methods to provide intact liver cells having a full complement of soluble enzymes such as aldolase and lactic dehydrogenase, it appeared that further studies on the use of PVP-sucrose for the isolation of liver cells might lead to a solution of this difficult problem. It was one of the objectives of the present project to explore the techniques of cell isolation in an attempt to isolate individual intact liver cells in high yield and to fractionate them according to cellular type for use in biochemical studies.

Part B included: NO

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Part 2: The soluble enzymes most readily lost from cells are those which also appear in the serum in large amounts in certain pathologic states. The association of elevated serum aldolase and lactic acid dehydrogenase with certain tumors and with certain lesions of muscle has been well documented in both humans and animals. Recently Riley (Science 132, 545, 1960) has reported the lactic acid dehydrogenase of mice was elevated markedly by the injection of cell free extracts. Because of the importance of these findings, we have carried out a collaborative study on serum lactic acid in animals injected with cell free extracts from a number of tumors of mice and rats not previously studied by Riley, and have extended our studies to the use of cell-free extracts which have been irradiated to destroy a possible viral agent.

Methods Employed:

Part 1: It was found that the number of isolated liver cells could be greatly increased by injecting trypsin intravenously before the animal was killed. The liver was removed 15 minutes after the trypsin was injected and was placed in 20% polyvinylpyrrolidone-10% sucrose, pH 7.0, at 0°. The liver was cut into 1-3 mm. squares, which then were suspended in the PVP-sucrose solution and centrifuged. The liver particles were resuspended in the PVP solution, drawn into and expelled three times from a 30 ml. syringe which had the orifice adaptor removed to give an opening with a 1.5 mm. diameter, and then were re-centrifuged. The precipitated liver particles were resuspended, syringed, and centrifuged a total of four times. Each time the number and vigor of the syringing was increased. The first two supernates contained little but red cells and cells damaged by the initial mincing. The last two supernates contained predominately single liver parenchymal cells. The final two supernates were combined and centrifuged for 10 minutes at 200 X G to separate the whole intact cells from any damaged cells or cell fragments. The cells were then resuspended in PVP-sucrose and stored at 0°.

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Attempts were made to substitute dextran or methylcellulose of various molecular weight for polyvinylpyrrolidone, but conditions have not been found which permitted the isolation of single fractions of intact cells by use of these polymers except in extremely low yield. Various methods for physically separating the tissue into cells have been studied. A homogenizer which permitted rather precise control of the distances between grinding surfaces was designed and constructed. Although this instrument is rather efficient in preparing homogenates of cell particulates, it has given somewhat lower yields of intact cells than the use of the syringe.

The activities of the enzymes aldolase, lactic acid dehydrogenase and catalase were determined on single cells at various periods after their isolation. The activities of these enzymes were also measured on cells which had been suspended in various tissue culture media. Preliminary studies on the overall metabolism have been carried out in collaboration with Dr. Mark Woods.

Part 2: The lactic acid dehydrogenase activities of the serum of normal animals and animals bearing various tumors have been carried out by measuring the decrease in the absorption of DPNH on the log-absorbance attachment of the Cary Recording Spectrophotometer. Rats and mice injected with cell-free tumor extracts and mice injected with irradiated tumor extracts have also been used as test animals.

Major Findings:

Part 1: A suspension of single cells was obtained which contained 15-25% of the total liver nitrogen when the in vivo injection of trypsin and the fractional centrifugation procedures described under methods were used. This fraction was relatively free of cell debris and was made up predominately of parenchymal cells. The staining properties of the cells would indicate that a majority of them are alive: i.e., over 90% of the cells in most suspensions did not take up trypan blue or nigrosin and nearly all of the cells took up tetrazolium under anerobic conditions.

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The ease with which single cell fractions could be isolated from other organs varied markedly. Cells could be isolated in high yield from spleen, thymus, lymphosarcoma, and hepatoma. Only after multiple trypsin injections could the kidney be separated into cells, and even then the yield of cells was smaller than that found for the preceding organs. Following a single injection of trypsin the kidney separated into golomeruli, and various lengths of tubules and blood vessels. Although muscle fibers which had been subjected to the in vivo trypsin injection could be easily teased apart, they had to be cut from the tendons. The brain and fibrous tumors separated into cells rather poorly, and the heart tissue did not separate at all.

Little change other than petechiae in thelungs could be seen on microscopic examination of the organs following a single in vivo injection of trypsin. The animals did not appear in pain and remained healthy if permitted to survive.

When liver cell suspensions were kept at 0° in 20% PVP-10% sucrose solutions, the activity of aldolase, lactic acid dehydrogenase and catalase over an 8 hour period was nearly the same on a nitrogen basis as that found in the liver of origin. When the isolated liver cells were suspended in culture media at 38°, certain of these enzymes were lost. In Earle's protein-free chemically defined media containing 20% PVP-10% sucrose, 80% of the lactic acid dehydrogenase was lost from the cells to the media in one hour while over 90% of the aldolase and the catalase could be recovered. Aldolase was rapidly lost when sucrose was removed from the media. The addition of methocel or serum did not prevent the rapid loss of enzymes.

Part 2: The previously reported findings by others that mice bearing tumors or mice injected with cell free extracts of tumors have markedly elevated serum lactic acid dehydrogenase activities was confirmed. However, rats injected with a long series of transplanted tumors had no such elevation, and cell free extracts of tumors were also without effect in these animals. When cell free extracts of mice were irradiated with a dose sufficient to inactivate most viruses, the elevation of lactic acid dehydrogenase was not prevented. This is

at least suggestive evidence that the factor causing the elevation of lactic acid dehydrogenase may not be a viral agent.

Significance to Biomedical Research: The ability to isolate cells from acult organs such as liver and kidney in high yield is sorely needed to permit studies on the degree of their heterogeneity and on the life span of the cells. If such a goal can be reached it will permit biochemical studies to progress down to the level of the histochemist working on intact tissues, and will enable the biochemist to study the characteristics of each type of cell within a given tissue.

The fact that adult liver cells lose a number of soluble enzymes during the process of isolation may account for the difficulty in maintaining these cells in tissue culture. The finding that certain high polymers, such as PVP, may prevent this loss provides a new approach to this difficult problem.

Proposed Course of Project:

1. In the next year, it is planned that further study be devoted to techniques for fractionating tissues into cellular components with special emphasis on density gradient centrifugation. Preliminary study using hepatoma cells have been encouraging.

2. It is planned that a comparative study of enzyme loss be made using isolated cells of embryonic tissue, adult cells and hepatomas.

Serial No. NCI-363
1. Lab. of Biochemistry
2. Enzymes and Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies on the Cachexia of Tumor-Bearing Animals.

Principal Investigator: Miloslav Rechcigl, Jr.

Other Investigators: Robert E. Greenfield

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1/2
Professional: 1/2
Other: None

Project Description:

Objectives: Previous studies from this laboratory dealt with the etiology and nature of anemia of tumor-bearing animals. These studies demonstrated that the anemia of rats with various tumors could be quantitatively correlated with the loss of cells by hemorrhage into the area of the tumor. In the course of this work it was observed that rats with marked anemia lost weight more rapidly, became cachectic and died while the tumors were in the range of 20-30% of the body weight, whereas in animals without anemia the onset of weight loss and cachexia was much slower, and the tumors frequently reached 50-65% of the body weight. However, in further study on the relationship of the anemia to the cachectic process, it was observed that during the period of most rapid tumor growth, and despite the onset of anemia, rats bearing the Lymphosarcoma R2788 gained appreciably more weight than the normal controls although they both had almost identical food consumption.

Part B included: YES

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In an attempt to determine whether the increased weight gain of tumor-bearing animals, when compared with the normal controls, resulted from an increased nitrogen retention or from accumulation of fluid, the following studies were undertaken. The nature of the body weight gain of the tumor-bearing animals was examined by the use of carcass and tumor analyses. The efficiency with which the animals utilized their diet was investigated by the use of metabolic balance studies.

Major findings: During period of most rapid tumor growth, rats bearing Lymphosarcoma R2788 gained more body weight than the control animals although their food consumption was almost identical. Rats bearing the Hepatoma 3683, on the other hand, consumed less food and grew at equal or slower rate than the controls. Both types of tumors, however, exerted similar effects on the host with regard to the development of anemia, elevation of plasma aldolase, depression in the liver catalase, adrenal enlargement, thymus involution and changes in weight of other organs.

Carcass and tumor analyses showed that the lymphosarcoma-bearing animals had an increased percentage of water sufficient to explain their increased weight gains. An equally high degree of hydration was observed in rats bearing Hepatoma 3683 whose body weight gains were lower than those of the normal controls. The combined nitrogen content of the tumor and the carcass of both groups of cancerous rats was similar to or smaller than the body nitrogen of the control animals, while the fat content of the tumor-bearing rats was markedly reduced.

In confirmation of these findings, balance studies showed nearly the same retention of nitrogen in rats bearing the Lymphosarcoma R2788 as in the controls, but there was a greater retention of water and sodium, concommitant with the increased weight gains in the tumor-bearing animals.

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During the terminal stages the lymphosarcoma-bearing rats were observed to suffer from anorexia, loss in total body weight and finally died. In this period the animals were in a negative nitrogen and potassium balance, the water retention was reduced below the normal level while the sodium balance was not appreciably affected. The positive sodium balance together with the decrease in the potassium retention indicate the possibility that, to a considerable extent, the terminal dehydration of the tumor-bearing animals was due to loss of intracellular fluid.

The onset of anorexia and the negative nitrogen balance was not prevented by increasing the sodium chloride intake of the animals. Moreover in other experiments in which the rats were given a choice of water and saline neither the tumor-bearing rats nor the control animals showed preference for fluid containing sodium chloride. A substitution of physiological saline for water in rats bearing the Lymphosarcoma R2788 resulted in greater fluid consumption, greater fluid retention and greater body weight gains than was observed with water ingestion. Consumption of saline did not cause any improvement in the nitrogen retention and in fact it resulted in greater loss of urinary nitrogen in both normal and lymphosarcoma-bearing rats when compared with the groups ingesting water. Ingestion of saline by the control and the hepatoma-bearing animals, while increasing the fluid consumption in both groups, had no apparent effect on the animals' body weight gain. Decrease in survival time among the tumor-bearing animals ingesting saline was also observed.

Significance to Cancer Research: This project is one phase of a program on the significance of anemia on the cachexia of the tumor-bearing host.

Proposed Course of Research: In order to gain a better understanding of the cachexia and various aspects of the tumor-host relationships it is planned to study the rates of synthesis and destruction of specific proteins within the tumor-bearing host. (See project NCI-320)

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Part B Honors, Awards, and Publications:

Publications other than abstracts from this project:

Rechcigl, M., Jr., Grantham, F., and Greenfield, R. E.:
Studies on the Cachexia of Tumor-Bearing Animals. I. Body Weight Changes, Carcass Composition and Metabolic Studies. Cancer Research, in press.

Serial No. NCI-367
1. Lab. of Biochemistry
2. Enzymes and Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Techniques for growing isolated tumors in vivo.

Principal Investigator: Pietro M. Gullino

Other Investigators: Flora H. Grantham

Cooperating Units: None

Man Years (calendar year 1960):
Total: 2
Professional: 1/2
Other: 1 1/2

Project Description:

Objectives: To develop a technique for growing a tumor isolated from the surrounding tissues and connected with the host only by one artery and one vein.

Method employed: A kidney was isolated from the surrounding adipose tissue and transferred to a pouch in the subcutaneous tissue of the renal region leaving the vascular peduncle as the only connection with the host. A tumor was then transplanted into the renal parenchyma and the kidney was enveloped in a bag of paraffin. The tumor grew in this bag, destroyed the kidney and the renal vessels remained the only connection between tumor and host. An ovary can be used instead of a kidney with equal success.

Part B included: NO

(ATTACHMENT I)

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Major findings: a) This technique provides the investigator with a growing tumor isolated from the surrounding tissues and connected with the host by a single artery and vein from which blood can be withdrawn. It can be applied to all tumors normally grown in rats, hamsters and mice. In rats ovarian implants grew up to the size of 14 g., namely about 400 times larger than the host ovary, renal implants grew up to 25 g., about 25 times the kidney's original weight.

b) Two transplants of the same tumor, one grown isolated in a paraffin bag and the other grown subcutaneously, showed the same correlation between wet weight, dry weight and total nitrogen and also the same level of anaerobic glycolysis.

Animals bearing the "isolated" transplant behaved in the same manner as subcutaneous tumors as to their change in body weight, food consumption and water intake. Liver catalase was also equally depressed. (A paper reporting this finding is in preparation).

Significance to cancer research: Since blood samples can be taken at intervals from the artery and vein, the material which enters or leaves the vascular system of the tumor can be quantitatively analyzed. Tumor tissue grown in the paraffin envelope is effectively separated from the surrounding tissues and any host cells entering the tumor must pass through the peduncle. With this procedure many problems of the tumor-host relationships can be more clearly defined and the requirements of the tumor more accurately determined. With further studies it is envisioned that tumors may be grown completely separated from the host by mechanically perfusing isolated tumors.

Proposed course: The fluid exchange between tumor and host will be the first problem studied with this procedure.

Serial No. NCI-368
1. Lab. of Biochemistry
2. Enzymes and Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A:

Project Title: Studies on the exchange of fluids between host and tumor.

Principal Investigator: Pietro M. Gullino

Other Investigators: Flora H. Grantham, Hosea E. Taylor

Cooperating Units: None

Man Years (calendar year 1960):
Total: 2
Professional: 1/2
Other: 1 1/2

Project Description:

Objectives: a) To calculate the blood flow of growing tumors. b) To analyze the response of the new formed vascular net of a tumor to stimuli of pharmacological and nervous origin. c) To look for a possible relationship between growth of the tumor and new formation of the vessel-bearing stroma.

Methods: a) The tumors have been grown isolated in a bag of paraffin (NCI-367). The single vein draining the tumor was cannulated and a direct measurement of the blood flow was performed for 30 minutes in anesthetized rats. Blood flow was also determined after administering drugs with adrenergic and cholinergic action or before and after removal of the coeliac ganglion.

b) Collagen was estimated by determining the hydroxyproline content of HCl-hydrolysates of tumors. Hydroxyproline was determined with the Beckman-Spinco amino acid analyzer, the colorimetric procedure of

Part B included: NO

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Prockop and Udenfriend has been added lately. A contamination of collagen from invaded tissues was avoided by growing the tumors in paraffin bags (NCI-367). The amount of collagen formed by the tumor was calculated by substracting the hydroxyproline content of the normal host organ (since the tumor implanted is one of a pair of organs like kidney or ovary).

Major findings: a) In an adult rat the blood flowing out of an ovary plus the oviduct has been measured as 12 ml./hour. The same amount of blood flows out of tumors weighing 6.0 g. or more, namely 200 times heavier than the ovary in which they were implanted. b) The growth of a tumor implanted in a kidney, which has a blood supply 20 times larger than the ovary, produces a decrease of the total blood flow to the degree that when the tumor has destroyed all the kidney parenchyma, the blood reaches values of the magnitude of those found in ovarian implants. c) Despite a difference of 20 fold in the blood supply between the ovary and the kidney, equal amounts of tumor implanted simultaneously in both organs of the same rat grew at the same rate, suggesting that a large blood supply does not increase the growth rate of rat tumors. d) Adrenalin reduces the blood outflow of a tumor and the removal of the coeliac ganglion has the same effect. Choline increases the blood outflow in some instances. f) the amount of collagen increases with the growth of the tumor. Although the number of determinations is not yet sufficient, it appears that this increase is proportional to the increase in the total nitrogen of the tumor.

Significance to Cancer Research: The determination of blood supply to a tumor appears to be a necessary step toward the solution of two fundamental problems in cancer research, to quantitate the metabolic needs of a growing mass of neoplastic tissue and to influence the tumor's growth with substances carried by the blood stream. The growth of a tumor is probably in some way dependent on the formation of new collagen because collagen forms a tridimensional net which connects the cells and participates in the diffusion of fluids.

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Proposed Course: An evaluation of the quantity and the chemical composition of the interstitial fluid of tumors will be attempted. Preliminary studies have been completed on the glutamic-oxalacetic transaminase activity, lactic acid dehydrogenase activity, catalase activity and aldolase activity of the blood going in and out of different type of isolated tumors grown as described in NCI-367.

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1. Lab. of Biochemistry
2. Enzymes and Metabolism Section
3. Bethesda, Md.

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Individual Project Report
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Part A:

Project Title: Studies on the Catalase Activity of Various Hepatomas.

Principal Investigators: Miloslav Rechcigl, Jr. and Vincent E. Price

Other Investigators: Harold P. Morris, Nutrition and Carcinogenesis Section, Laboratory of Biochemistry, NCI
Herschel Sidransky, Laboratory of Pathology, NCI

Cooperating Units: None

Man Years: (calendar year 1960)
Total: 1-1/2
Professional: 1/2
Other: 1

Project Description:

Objectives: Tumors in general have a low level of catalase activity. This is consistent with the postulate that a substance, often referred to as toxohormone, is produced in tumor cells which passes to the liver causing a reduction in the catalase of that organ. If such a substance is produced in tumor cells it would presumably have a direct effect on the catalase of the tumor cell. In view of the reports from Potter's laboratory that the Morris 5123 Hepatoma has fairly high levels of a number of enzymes a study has been made of the catalase activity of the 5123 tumor and a number of other hepatomas to see if catalase is present or absent in these tumors.

Part B included: NO

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Major findings: A fairly high catalase activity was found in the 5123 Hepatoma and in the primary and the transplanted ethionine-induced hepatomas of rats. A high activity was also found in spontaneous hepatomas of mice. Almost no activity could be demonstrated in the Dunning, Novikoff and the 3683 hepatomas of rats.

Despite the fact that fairly high levels of catalase were found in the tumor, the catalase activity of the liver and kidneys of animals bearing 5123 and ethionine-induced hepatomas was depressed to a degree comparable with the other tumor-bearing animals tested. The amount of depression was correlated with the size of the tumor and the loss of the carcass weight.

These data indicate that a depression can be observed in the catalase activity in the liver and kidneys of the tumor-bearing host even in the presence of significant levels of catalase activity within the tumor itself. These observations throw some doubt on the concept of toxohormone as causing the depression of catalase in tissues of the host, since it seems unlikely that such factor would depress the enzyme level of the liver and kidneys without drastically affecting the catalase of the tumor itself. There remains a small possibility, however, that toxohormone is not released in an active state within the tumor cell, which would account for its failure to depress the catalase activity of certain hepatomas.

In view of the lowering of the liver catalase of normal animals following the feeding of a protein-free diet (as described in NCI-320, 1960) it was of interest to find out whether a comparable decrease in the enzyme activity would occur in Hepatoma 5123. The experiments have shown that a protein-free diet produces no significant lowering of catalase in the 5123 Hepatoma, even though the catalase of the liver was markedly reduced.

Significance to Cancer Research: One of the classical effects of a tumor on its host is the production of a marked lowering in the liver catalase. It has been postulated that this effect is produced by a substance called toxohormone. In recent years substances have been isolated from tumors by Nakahara in Japan and by Yunoki and Griffin in this country, which do

produce a reduction in catalase activity when injected into normal animals. Although such substances can be isolated from tumors, the crucial question is whether they are released from the tumor cells into circulation. Data on this point have been very sketchy and unsatisfactory.

In the meantime studies by Greenfield and Price (Acta Unio Intern. contra Cancrum 15: 888-892, 1959) and by Rechcigl and Price (NCI-320, 1960) have pointed out, respectively, that hemorrhage and a protein-free diet can produce marked lowering of the liver catalase, thus suggesting other mechanisms that might be acting in the tumor-bearing animal to lower the liver catalase.

The data reported above point out that some hepatomas have a very considerable amount of catalase, although they produce a lowering of liver catalase comparable to that of other tumors. These findings would tend to oppose the toxohormone theory and to support the possibility that some other mechanism, such as a deficiency in some of the amino acids essential for protein synthesis, may cause the reduction of catalase in the host liver.

Proposed Course of Research: Since there are significant levels of catalase in the Morris 5123 Hepatoma and in the ethionine-induced hepatomas, it will be of interest to study the rates of synthesis and destruction of catalase in these tumors. In view of the question as to whether proteins are being turned over during the logarithmic growth of cells we are particularly curious to see if the rate constant for catalase destruction is similar to that of the liver. To our knowledge no one has studied the in vivo rates of synthesis and destruction of a apecific protein within a tumor.

We would also like to study various factors which may change the rate of catalase synthesis and destruction in the tumor and to compare them with the effect on the host. It was noted in the present work that a

protein-free diet produced a marked lowering in the catalase activity of the liver but had no effect on the 5123 Hepatoma. The question arises as to whether the tumor will be equally resistant to other nutritional and hormonal changes in maintaining its level of catalase activity.

Transplantation of a primary ethionine-induced hepatoma resulted in tumors having two different levels of catalase activity, one of which was twice that of the normal liver, whereas the other was about half the normal level. The rates of catalase synthesis and destruction will be measured in subsequent generations of both of these tumor lines to find out whether the variable levels result from alterations in synthesis or destruction and to correlate these observations with the histological changes within the tumor.

Serial No. NCI-370
1. Lab. of Biochemistry
2. Enzymes and Metabolism Section

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: The Sulfhydryl Dependence of the RNASE Activities of Liver.

Principal Investigator: Mary E. Maver

Other Investigators: Antoinette E. Greco

Cooperating Units: None

Man Years (calendar year 1960)
Total: 1-1/2
Professional: 1/2
Other: 1

Project Description:

Objective: To determine whether the RNase activities of normal rat liver are dependent upon sulfhydryl groups for their activity.

Methods employed: The effects of p-chloromercuribenzoate (CMB) upon the acid and alkaline RNase activities of rat liver homogenates and crude dialyzed extracts are being studied. The action of CMB is also being followed using the peak tube activities obtained by chromatographic separation of these activities on DEAE and CM. After the CMB has combined with the sulfhydryl groups on the RNase proteins, excess cysteine or glutathione is added and the RNase activities are assayed again. In other experiments, purified fractions of RNase activities obtained by chromatographic fractionation of more active nuclease preparations are treated with CMB. Simultaneously the degree of inactivation of the RNase is assayed and the combination of CMB with the SH on the RNase protein is followed by the spectrophotographic technique of Boyer.

Part B included: NO

(ATTACHMENT I)
Serial No. NCI-370

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Major findings: As has been shown by others, CMB activates the alkaline RNases by combining with an inhibitor which is present as an impurity. When this impurity has been removed by the chemical purification procedures used to obtain our more active nuclease preparations, often the alkaline as well as the acid RNase activities can be activated (about 20%) by cysteine, and inhibited by CMB. The acid RNase is much more sensitive to CMB than the alkaline RNases. The inhibitions of RNase activities by CMB can be reversed by excess cysteine when the right quantitative conditions prevail. Spectrophotometric assays according to Boyer show that the combination of the SH of the RNase protein with the CMB is directly correlated in timed reactions with the inactivation of the enzyme.

Significance: It is recognized that the majority of intracellular hydrolases are dependent upon free sulfhydryl group s for their catalytic activity. The significance of this study probably lies in the unique role of the RNases in providing RNA for protein synthesis, and in liberating energy yielding, or accepting, nucleotides. Intracellular oxidation-reduction conditions could regulate the activity of these RNases throughtheir sulfhydryl groups.

Proposed Course: Several RNase fractions which have been purified by chromatography on DEAE and CM will be examined for their dependence on SH groups for activity. Plans have been made to determine the molecular weights of the RNase proteins in order that the number of SH groups present per molecule can be determined.

Serial No. NCI-324
1. Lab. of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Biological Studies of the Mechanism(s) of Carcinogenesis

Principal Investigator: H. P. Morris

Other Investigators: B. P. Wagner, H. L. Stewart, H. Sidransky and K. Snell

Cooperating Units: Dr. C. A. Velat, Providence Hospital, Detroit, Michigan (formerly Pathology Branch, NCI) and F. E. Ray, Cancer Research Laboratory, University of Florida, Gainesville, Florida.

Man Years (Calendar Year 1960):
Total: 2-1/3
Professional: 2/3
Other: 1-2/3

Project Description:

New knowledge concerning the mechanism(s) of carcinogenesis in several species of animals and by different routes of administration. The carcinogenic activity of N-2-fluorenyl-acetamide (2-FAA) and related analogues, derivatives, and metabolites and/or related compounds are used. The program envisages research in depth on the carcinogenicity of 2-FAA and its metabolism (cf. projects 325, 327, 346, 347 and 349, for other aspects of the program). The discovery of differences in carcinogenic activity related to structure or metabolism, or as influenced by vitamins or hormones are being searched for, in the belief that such differences can be used to further a better understanding of the causation of cancer by chemical carcinogens.

Methods: Highly purified chemical compounds are administered to several species of animals by oral, intraperitoneal or by other routes. Dogs, rats, hamstera and mastomys are the experimental species most commonly used. The period of

Part B. included: YES

administration varies from a few weeks to several years depending on the species and objectives. Records of body weight, food and drug intake are made at suitable intervals. At the termination of an experiment, careful gross observations are noted. The tissues fixed, sectioned by tissue laboratory, and the resulting microscopic slides are examined by a pathologist in order to fully evaluate the effects of the treatments.

Major Findings: (a) As reported last year, mostly from gross observations, the four metabolites of 2-FAA namely: 5-OH, 7-OH, 3-OH, and 1-OH-2-FAA were much less carcinogenic than 2-FAA itself. These compounds are formed in vivo and excreted in the urine in largest amounts - almost entirely as conjugates of glucuronic and sulfuric acids (cf project 327). These facts seem to point to the probability that hydroxylation and conjugation are metabolic processes concerned with detoxication and excretion. During the metabolism, whether by action on cell constituents at sites remote from the portal of entry or whether by combining with essential metabolites required for normal cell division or by some other mechanism as yet unknown, the chemical carcinogen appears to alter the genetic-information-transmitting-mechanism of one or more cells to such an extent that it (they) no longer respond(s) to the host control mechanisms concerned with cell division and multiplication.

A chemical carcinogen which is slow acting e.g. N-(2-fluorenyl) phthalamic acid (2-FPA) has induced a transplantable, malignant liver neoplasm (Morris No. 5123) which has many enzymic characteristics similar to normal liver and appears more like normal liver than any transplantable rat liver tumor studied to date. The metabolism of 2-FPA (cf.325) as studied by colorimetric methods indicated that in vivo 2-FPA is hydrolyzed and then acetylated to 2-FAA yet the action of the two compounds in inducing cancer of the liver is (at least timewise) vastly different. The liver tumors induced by FPA occurred 8 months after the drug had been removed from the diet while rats receiving 2-FAA have died of tumors by 10 or 11 months. One interpretation would be that 2-FPA produced a very slight alteration in the genetic transmitting information portion of the liver cell sufficient for it to develop, during a long period of time, independently of host control. Such an alteration could be some sort of enzymic deletion which permitted the altered cell(s) to grow in the primary host liver for many months without causing the death of the animal.

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(b) Carcinogenic activity of compounds related to 2-FAA 2,7-FAA first shown in this laboratory a decade ago, to be a very active carcinogen has been further studied and compared with 2-FAA. (cf Dr. Stewart and Snell). Four papers setting forth the observations have been submitted for publication. Some of the most important findings in rats, include tumors of the small intestine, gastric portion of the rat stomach, salivary gland tumors and neurogenic tumors. This is the first induction, to our knowledge, of gastric carcinoma by an orally administered carcinogen. This provides a new laboratory tool to investigate a type of cancer of great importance in man. Another important observation found in the rats that ingested 2,7-FAA was extensive atrophy of many organs and tissues. This poses the problem of determining the role of atrophy in the mechanism(s) of carcinogenesis.

It was reported this year [Part B Ref. (1)]that N-2-fluorenyl-2,2,2-trifluoroacetamide was an exceedingly active carcinogen. The influence of the substitution of the hydrogens on the ω-carbon atom by the halogen fluorine has been extended to the 2,7-FAA in which all six of the ω-carbon hydrogens were replaced with fluorine. The substitution of the halogen fluorine in substituents in both the 2 and 7 positions of the fluorene molecule from gross observations has increased the carcinogenic activity. Of sites most influenced by the alteration of the structure of the fluorene molecule from that of 2,7-FAA were the mammary glands and salivary glands where the alteration caused an increased activity. The substitution of the halogen fluorine for hydrogens in the ω-carbon of 1-FAA has also been found to increase the activity of this relatively inactive carcinogen to one of moderate activity. Here again the activity was most evident toward the mammary gland and salivary gland.

(c) The animal experiments concerned with the effect on carcinogenic activity brought about by blocking the most active hydroxylation positions of a simple aromatic amine such as analine or acetaniline have been completed. Blocking both the ortho positions and the para position with CH_3, Cl and F were studied and the blocking of the para position by a hydroxyl was also studied. The 2,4,6-trifluoroacetanilide animal experiment has not yet been completed. Liver tumors, from gross observations, were induced in rats ingesting 2,4,6-trimethylaniline where the three most readily hydroxylated positions were blocked. Not only liver tumors were induced but a severe

cholangiofibrosis in a number of rats occurred, and enlarged pituitary glands were noted. In the p-hydroxyacetanilide experiment mammary glands were hypertrophied and a large number of enlarged pituitary glands were found at autopsy. Enlargement of the pituitary gland was observed in female rats ingesting acetanilide. Pituitary enlargement was also noted in rats ingesting 2,4,6-trichloroacetanilide.

(d) Two groups of dogs were continued on diets containing N-(2-fluorenyl)-2,2,2-trifluoroacetamide and 4'-fluoro-4-acetylaminobiphenyl-compounds highly carcinogenic to the rat. One dog receiving the former compound died during the year without showing any gross tumors.

(e) Syrian hamster animal experiments have been completed. Diets containing 2-FAA, 2,7-FAA, and 2-FdiAA were used. The microscopic material is not ready for study.

(f) Route of administration: Since in some metabolic studies the 2-FAA had been administered by the intraperitoneal (I.P.) route it was important to determine the comparative carcinogenic activity of the oral vs the I.P. route of administration. Tumors were induced in female Buffalo-strain rats after I.P., administration of 2-FAA in gum acacia suspension in the mammary gland, liver and a few other sites. The administration of 2-FAA in gum acacia to female Buffalo-strain rats by stomach tube also resulted in mammary tumors, liver tumors and tumors at a few other sites. Only about half as much 2-FAA was administered by the oral route as by the I.P. route. The duration of the experiment was shortened in the stomach feeding group because of technical difficulties of administering the 2-FAA by ridgid stomach tubes with resultant loss of a high percentage of the animals. A modification of the technique by use of flexible stomach tubes decreased mortality materially but not before many animals had been lost. It is unwise from the present data to conclude that 2-FAA in a gum acacia suspension is a more active carcinogen by the oral vs I.P. route of administration. The I.P. route is not a satisfactory method of administering the carcinogen because much damage and inflammation occurs in the abdominal cavity prior to the development of tumors and it is impossible to carry out a satisfactory autopsy. The I. P. injection of 2,7-FAA in a gum acacia suspension into rats has been completed. The biological material has not been studied histologically but from gross

observation mammary tumors, liver tumors, ear duct tumors, intestinal tumors (no stomach tumors were macroscopically seen) and a few tumors at other sites were noted. The I.P. animal experiments were of shorter duration than those from oral ingestion of 2,7-FAA. A final comparison of the effectiveness of the oral route of administration vs the intraperitoneal route will have to await the histological examination of the microscopic slides. One objective of the experiment was attained, however, in that tumors of the intestine and gastric tumors following microscopic examination were induced by the I.P. route without the 2,7-FAA being introduced directly into the G.I. tract.

(g) Transplantable hepatoma 5123. This neoplasm induced in a rat liver following ingestion of N-2-fluorenylphthalamic acid has been studied in 20 different laboratories during 1960. This tumor which was induced in an inbred strain of rat (Buffalo) available only at N.I.H. has necessitated a large operation in making the tumor available to those investigators who have requested the tumor and who have research underway in which studies of this tumor would make a valuable contribution to our knowledge of cancer. A total of 454 rats were inoculated with No. 5123 hepatoma during this calendar year. Many of the results of these studies are not yet available but preliminary reports indicate this tumor is more nearly like normal liver in many enzymic activities than any other transplanted hepatoma so far studied. Some enzymic activities, however, are much higher in the tumor than found in normal liver. Four sublines of this tumor were established and extensive records are being kept to see if any changes occur in rate of growth or in enzymic activities of these sublines. Tumor No. 5123 seems to grow somewhat more rapidly in male rats of each of the four sublines than in females. Other inoculations of primary chemically induced liver tumors have been made in the hopes of finding valuable new enzymic characteristics of other transplantable hepatomas. No information is available on these tumors to date.

Significance: (a) Our present knowledge of the metabolism of 2-FAA supports the view (expressed last year) that hydroxylation and conjugation are metabolic processes concerned with detoxication and excretion of the carcinogen. We have not yet been able to characterize the mechanism(s) involved in the chemical carcinogenic process.

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(b) Variations in chemical structure has induced tumors at different sites and had yielded results which are influenced by vitamin B_6 and by hormones. These differences are being used as sign posts for further studies on carcinogenesis.

(c) The blocking of the usual sites of hydroxylation of simple aromatic amines has yielded information which suggests that interference with this process may increase carcinogenesis in some instances at least.

(d) The introduction of a transplantable liver tumor, No. 5123 in this section has greatly increased our knowledge of rat liver cancer and holds promise for most significant future developments. The enzymic evidence on the thymine catabolism of this tumor suggests that it is derived from a parenchymal liver cell. Furthermore, the studies so far completed suggest that extreme caution must be used in the interpretation that there is "a convergence of enzyme patterns to a single type" in the development of liver cancer since the enzymic patterns of No. 5123 are so much like those of normal liver. The importance of inducing tumors by chemical carcinogens into inbred strains of rats is especially stressed because slow growing tumors may be obtained and transplanted whereas such tumors might not survive in non-inbred rats and would be permanently lost.

Proposed Course: Completion of experiments now underway, analysis of the results and their preparation for publication. New experiments concerned with factors involved in the induction of gastric carcinoma by oral ingestion of a chemical carcinogen are under way.

The induction of transplantable liver tumors in inbred rats by several different chemical carcinogens and a study of their enzyme activities in order to better understand the carcinogenic mechanism(s).

(ATTACHMENT NO. VI)
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Part B: Honors, Awards and Publications

Publications other than Abstracts from this project:

(1) Morris, H. P., Velat, C. A., Wagner, B. P., Dahlgard, M., and Ray, F. E. Studies of Carcinogenicity in the rat of derivatives of aromatic amines related to N-2-fluorenyl-acetamide. J. Nat'l Cancer Institue 24: 149-180 (1960)

(2) Morris, H. P., Sidransky, H., Wagner, B. P. and Dyer, H.M. Some Characteristics of Transplantable Rat Hepatoma No. 5123 induced by ingestion of N-(2-fluorenyl)phthalamic acid. Cancer Research 20: 1252-54, 1960.

(3) Potter, V. R., Pitot, H., Ono, T., and Morris, H. P. The Comparative Enzymology and Cell Origin of Rat Hepatomas. I. Deoxycytidylate Deaminase and Thymine Degradation. Cancer Research 20: 1255-1261, 1960.

Serial No. NCI-325
1. Lab. of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Some Biochemical Aspects of Experimental Cancer: I. Interference with Vitamin B_6 Activity of Several Hepato-carcinogenic and Hepato-noncarcinogenic Aromatic Amines. II. Studies of Glutamic-oxalacetic Transaminase (GOT) Activities with Relation to Cancer; (1) comparison of transaminase activity of the sera of rats fed some hepatocarcinogenic and some hepato-noncarcinogenic aromatic amines; (2) comparison of GOT activities of sera and of livers and tumors of rats bearing transplants of several different liver tumors, including Morris No. 5123 and of one mammary and one carcinosarcoma.

Principal Investigator: Helen M. Dyer

Other Investigators: B. Ensfield and H. P. Morris

Cooperating Units: P. Gullino, Enzymes and Metabolism Section, Laboratory of Biochemistry, NCI

Man Years (calendar year 1960):
Total: 2
Professional: 1
Other: 1

Project Description:
Objective: To find some biochemical activity or activities that are characteristic of "precancerous" or of cancer tissue.

Part B included: YES

(ATTACHMENT I)
Serial No. NCI-325

PHS-NIH
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Methods Employed: Spectrophotometric and paper chromatographic methods for measurements of N-2-fluorenylacetamide (2-FAA) and its metabolites and of related compounds have been described in previous reports, also methods for metabolites of L-tryptophan. In addition, the following methods have been used, with or without modification: (1) For the glutamic-aspartic transaminase activities of livers and tumors, Tonhazy, White, and Umbreit, Archives of Biochem. 28:36, 1950. (2) For transaminase activity (GOT) of blood serum or plasma, Steinberg, Baldwin, and Ostrow, J. of Lab. and Clin. Med. 48:144, 1956.

Major Findings: Project I: Further investigation of the observed interference of 2-FAA and of related compounds with the metabolism of a challenging dose of L-tryptophan has led, by analogy to a logical theory of the mechanism of the effect. A final report of the study has been accepted for publication. The effect observed, namely the excretion of increased amounts of urinary xanthurenic acid, kynurenic acid, and kynurenine derivatives after a load dose of L-tryptophan to rats maintained on carcinogenic concentrations of 2-FAA is due most likely to an inhibition of kynureninase activity. The inhibitory effect may result from combination of the coenzyme pyridoxal phosphate (PP) with fluorenamine or some other amino metabolite of 2-FAA in the form of a Schiff base. A further decrease in available free PP may occur as a result of the accumulation of kynurenine from the load tryptophan. It has been reported that kynurenine as well as other aromatic amines tested cause a decrease in available PP activity in Neurospora crassa and the evidence indicated that a Schiff base reaction was responsible. The findings in our studies support the experimental evidence that more than the ordinary requirement of B_6 may be needed by rats ingesting aminocarcinogens. A relatively low concentration of dietary B_6 was found to cause an increase in the period of induction and/or a lower incidence of tumors of the liver induced by 2-FAA and by p-dimethylaminoazobenzene (cf. project 324). Thus, the action of 2-FAA in reducing the amount of B_6 available in the rat would tend to retard rather than promote its own hepatocarcinogenic activity.

In some cases the degree of the effect of some tested aromatic amines on load tryptophan metabolism paralleled their hepatocarcinogenic activities, for instance: no effect of the non-hepatocarcinogen-N-(7-hydroxy-2-fluorenyl)acetamide and a strong effect of N,N-2-fluorenyldiacetamide which is a very active hepatocarcinogen; 4'-fluorine-4-biphenylacetamide, a potent liver carcinogen, had a greater effect on load-tryptophan metabolism than the related non-hepatocarcinogen 4-biphenylacetamide; and finally, there was a slightly greater effect of the hepatocarcinogen 2-(4'-methyl)-fluorenylbenzoyl-amide than of the non-hepatocarcinogen 2-(3'-methyl)-fluorenylbenzoyl-amide. After feeding for 2-3 weeks, and again after 4.5 weeks N-(2-fluorenyl)-2,2,2-trifluoroacetamide, a potent hepatocarcinogen, had a severe effect upon tryptophan metabolism. N-(2-fluorenyl-2-fluoroacetamide however, which proved to be too toxic for survival of the rats for a final comparison of its carcinogenic effect, did not interfere with load tryptophan metabolism between 2 and 3 weeks but did have an effect after 4.5 weeks of feeding when the animals were in poor physical condition. The differences in effects of these related pairs of aromatic acetylamides may reflect the rate at which the various compounds yield free amines and the amount of the free amine that comes into contact with pyridoxal phosphate. Carcinogenic activity of the hepatocarcinogenic member of each pair may be involved only indirectly as a result of poorer nutrition due to combination of an amino metabolite causing a decrease in free pyridoxal phosphate, but the failure of the non-hepatocarcinogenic compounds to induce liver tumors apparently is not a result of their combining with pyridoxal phosphate.

In summary, there is evidence that some aromatic amino carcinogens produce liver tumors in a shorter time when adequate, rather than limited amounts of vitamin B_6 are available. Some other B factors have not been found to influence so readily the incidence and induction time of liver tumors caused by feeding 2-FAA. The need for an abundance of dietary B_6 in carcinogenesis of aromatic amines is probably due to Schiff base type reaction between the amines and pyridoxal phosphate causing a decrease in available free coenzyme.

(ATTACHMENT III)
Serial No. NCI-325

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Project II: No effect was observed on the concentration of GOT of the serum of young adult male Buffalo rats fed one to two months diets containing carcinogenic levels or molecular equivalent amounts of 2-FAA, 7-OH-2-FAA, 4-biphenyl-AA, 4'-F-4-biphenyl-AA, 2-(4'-methyl)-fluorenylbenzoylamide, 2-(3'-methyl)-FBA, N-(2-fluorenyl)-2, 2, 2-trifluoroacetamide and N-(2-fluorenyl)2, fluoro-acetamide. Thus it appears that neither the hepatocarcinogenic aromatic amines nor the chemically related non-hepatocarcinogenic compounds in the dosage used for the induction of tumors, has any effect on the level of serum transaminase of male Buffalo rats during the first 2 months of feeding.

Data from the following GOT studies are being incorporated at this time in a paper for publication and in abstract form for consideration at the Spring Meeting of the American Association for Cancer Research. A summary of the results is included here.

Materials: 1. Subcutaneous, intraperitoneal, intramuscular and/or ovarian and kidney transplants of liver tumors Morris 5123, Morris 3683, Sidransky-ethionine induced, Novikoff, and Dunning, and Morris mammary tumor 6338, and Walker carcinoma-sarcoma 256 were also used.

2. Male and/or female rats of the Buffalo, Holtzman, Sprague-Dawley, Fischer, Irish (AxC), and Osborne-Mendel strains were used. The sera and homogenates of livers of control rats were analyzed for comparison of the transaminase activities of the sera, liver and tumor tissue of the tumor-bearing rats.

Results: 1. The moisture content of liver tumors was always greater than that of the host's liver, with averages of 80% for tumor and 70% for liver.

2. The average total nitrogen content of transplants of liver tumors was 70 to 81% that of liver of controls or host livers on a wet weight basis, but on a dry weight basis the tumors varied from 111 to 116% as much nitrogen as that of the livers.

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3. The transaminase activities of the sera of all normal rats were remarkably constant varying from 113 to 185 units per ml. with an average of 138 ± S.D. 4.2. Some of the higher values were from male rats, but not invariably.

4. The transaminase activities of the livers of normal rats varied from 541 to 898 units per mg. dry wt., average 667 ± 22. Transaminase activites of the livers of animals bearing tumor transplants did not differ significantly from those of normal animals.

5. The transaminase activity of Morris hepatoma 5123 was significantly higher than that of host livers on the basis of N content, wet or dry weight. On a dry wt. basis the tumors contained 2 to 6 times as many units as liver. The Novikoff, Dunning, Sidransky-ethionine, and Morris 3683 liver tumors, and mammary carcinoma 6338 and the Walker carcinoma-sarcoma contained one-fourth to one-third the amount of transaminase activity of normal liver.

6. GOT activity of the sera of rats bearing Morris hepatoma 5123 was 4 to 25 times (400 to 2500%) that of normal rats whereas the sera of rats bearing the other transplanted tumors contained approximately the following average percentages of GOT of normal rats: Novikoff and Sidransky transplanted liver tumors, 150%, Morris mammary 6338, 200%, and Morris hepatoma 3683, Dunning hepatoma, and Walker carcinoma-sarcoma, 300%.

7. No differences in activities, moisture, or nitrogen contents were found in subcutaneous, intraperitoneal, intramuscular, ovarian and kidney transplants of the same tumor.

8. In Morris tumor 5123 the size of the tumor did not seem to influence the concentration of activity present in the tumor tissue, but the amount of serum GOT paralleled the amount of tumor tissue present in the animal. When the tumor was removed surgically the elevated serum GOT activity dropped rapidly in 24 hours and reached normal values in approximately 48 hours. Five to seven days following tumor removal the concentration of serum GOT was usually less than that of normal rats.

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9. Intraperitoneal injection into normal rats of 65,000 to 130,000 units of GOT in the supernatant layer of a centrifuged homogenate of hepatoma 5123 raised the GOT concentration ofserum collected from the animal after 6 hours to approximately the maximum elevation observed under any of the conditions studied (around 3000 units per ml.). Serum GOT had dropped significantly 24 hours after the injection of GOT and was only slightly elevated, if at all, at 48 hours.

10. Studies are being made of the urinary excretion of GOT. Very small amounts of activity have been found in the urine at any time. Sometimes, but not always, the urinary excretion of GOT in 24 hours is 50 times greater for rats bearing transplants of tumor 5123 than of normal rats, but the greatest amount found to be excreted in 24 hours was 2000 units by a rat whose serum GOT was 2700 units per ml. Three to five days after tumor removal urinary GOT was found to be back to the low of the non-tumor rats. Earlier periods were not tried with those rats because of the possible effect of surgery. We are now comparing the decrease of both serum GOT and urine GOT in rats following i.p. administration of tumor GOT in an effort to see whether most of the activity is excreted by the kidneys or destroyed in the animal. The evidence is that the activity that disappears from the blood is not found in the urine. However, when tumor GOT is added to urine and refrigerated the activity appears to be stable for days.

The results of the urine and serum analyses to date suggest that the increased serum GOT activity originates in the tumor tissue and that it accumulates in the blood because it can only be excreted slowly by the kidneys or perhaps must be destroyed in the body.

Significance to bio-medical research and the program of the Institute: It is hoped that some useful information of specific properties of malignant tissue will result from the accumulation of data on the comparative metabolic behavior of (1) cancer tissue and the non-cancerous tissue of origin or (2) of a target organ during the administration of a carcinogen. The results have been most often disappointing affording only a mite of satisfaction when a logical

mechanism is found to account for some of the experimental observations.

For example, in the investigation of the interfering effect of 2-FAA on the metabolism of a load dose of L-tryptophan, the initial plan was to find out why the 2-FAA fed rat developed only liver tumors in the absence of supplementary tryptophan, whereas both a higher incidence of liver tumors and 100 percent incidence of bladder tumors appeared in rats fed 2-FAA and 1 to 2% L-tryptophan in the diet. The effect of 2-FAA upon tryptophan metabolism was of special interest because this amino acid or some of its metabolites have been found to play a role in the stimulation of growth of plant tissue and have been implicated in clinical as well as in experimental bladder cancer. The evidence reported by us that 2-FAA and probably other aromatic amino carcinogens may combine chemically with pyridoxal phosphate and so reduce the availability of the free coenzyme suggests that the retardation in development of 2-FAA and dimethylaminoazobenzene induced tumors in rats fed diets low in B_6 may reflect the effect of a poorer state of nutrition. The appearance of bladder tumors in addition to an increased incidence of liver tumors in rats fed diets containing 2-FAA and supplementary tryptophan may be due to the lengthening of the period of induction as a result of a mild B_6 deficiency caused by 2-FAA and by kynurenine accumulation from the extra tryptophan and resulting in longer survival of the animals. An increase in the time of survival of the rats has been reported.

The significance of finding much more transaminase activityin transplants of the slow growing liver tumor Morris 5123 than in livers of the host, and only traces of activity in transplants of the fast growing Novikoff and Dunning liver tumors would suggest that this enzyme activity does not have to be present in large amounts for the rapid growth of cancer tissue, unless it is possible that the enzyme is dissipated (used up) in the fast growing tissue as fast as it is synthesized. One logical interpretation of the comparative findings is that the cell(s) of origin of the several liver tumors is different. Thus the Morris tumor 5123 may have arisen from liver cells that synthesize transaminase whereas the Novikoff and Dunning and Ethionine-induced tumors arise from liver cells that make less or none of this enzyme.

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A possible difference in cells of origin of the tumors studied here is suggested by the investigations of other enzyme activities in other laboratories. It is interesting that many of the enzyme activities are missing or low in those tumors that grow the fastest. This last finding could throw the emphasis on the relation between enzyme activities and degrees of malignancy rather than on the carrying over of enzyme functions typical of the cell(s) of origin.

Proposed Course of Project: Before terminating the GOT studies, it may be of value to compare GOT activities of the sera of cancer patients with and without evidence of cachexia with that of suitable control individuals who have no evidence of cancer. Regenerating liver, and pregnancy might be interesting to study with regard to transaminase activities of the liver and the sera of the animals, especially if it is found that cancer patients, like the rats bearing tumor transplants show elevated serum GOT. Surprisingly little is found in the literature concerning GOT, except/an increasing number of papers indicating that liver damage and cardiac infractions are associated with increased GOT of the serum. In his review article Meister mentioned the probable significance of comparing transaminase activities of different tumors.

Plans for the coming year include a continuation of efforts to learn more about the mechanism of carcinogenesis of some fluorenamine compounds. The metabolism of N, N'-2, 7-fluorenyldiacetamide in the rat will be studied closely to seek a possible explanation for its unexpected carcinogenic action in the gastric mucosa and the intestinal tract. The possibility of di-N-OH or mono-N-OH metabolites and the site of their occurrence will be of particular interest in view of the Millers' finding that the N-hydroxy-metabolite of 2-FAA is more carcinogenic than the parent substance.

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Part B. Publications

1. Dyer, Helen M. and Morris, Harold P. An effect of N-2-fluorenylacetamide on the metabolism of tryptophan in rats. Accepted for publication in February, 1961. J. N. C. I.

2. Morris, H. P., Wagner, B. P. Sidransky, H. and Dyer, H. M. Some characteristics of transplantable rat hepatoma No. 5123 induced by ingestion of N-(2-fluorenyl)-phthalamic acid. Cancer Research 20: 1252-1254, 1960.

3. Obituary of Dr. Carl Voegtlin, by Helen M. Dyer (unsigned) J. Nat. Cancer Inst. 25: V-XV, 1960.

Serial No. NCI-327
1. Lab. of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Maryland

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Part A

Project Title: Mechanism of Carcinogenesis - Chemistry and Metabolism of N-2-Fluorenylacetamide and Related Compounds.

Principal Investigators: J. H. Weisburger and E. K. Weisburger

Other Investigators: K. Suzuki (Visiting Scientist) P. H. Grantham and H. P. Morris

Cooperating Units: Dr. M. S. Burstone, LHP, NIDR, NIH (Serial No. NIDR-16)

Man Years (calendar year 1960):
Total: 4-1/2
Professional: 2-1/2
Other: 2

Project Description:

Objective: In order to gain an understanding of the origin of cancer, phenomena relating to the initiation and development of neoplasia are studied in animals by means of synthetic chemical carcinogens such as N-2-fluorenylacetamide. A number of approaches are followed: 1. The metabolism of the carcinogen, or of a related non-carcinogen, is investigated in susceptible and resistant species. 2. The interaction of the chemicals with body constituents is studied in order to ascertain the factor(s) involved. 3. Biochemical, organic-chemical, and physical-chemical studies on model systems related to the carcinogen are performed to attain an understanding of the fundamental factors involved in chemical carcinogenesis.

Part B included: YES

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Methods: 1. Radiochemical and microtechniques are applied to isolate and determine metabolites of N-2-fluorenylacetamide and related compounds. 2. In a few cases biosynthetic methods are used to prepare isotopically labeled compounds for metabolism studies when other procedures do not yield the desired materials with equal ease or efficiency. 3. Chemicals required as standards for metabolism studies, or for histochemical demonstration of certain intracellular enzymes, are synthesized by methods developed for the purpose. 4. Research on analytical, chromatographic, and physical-chemical procedures is performed in support of these investigations.

Major Findings: I. Biochemical Aspects of Problem.

1. Metabolism of N-2-fluorenylacetamide in rats.
A. Urinary metabolites. Our development of a method for the separation of the urinary metabolites of the carcinogen N-2-fluorenylacetamide was reported in 1957. Further study of the metabolites was performed by this technique with particular emphasis on the sulfuric acid conjugates. Briefly, a urine extract chromatographed on alumina was partitioned into unconjugated compounds, sulfuric acid conjugates, and glucuronic acid conjugates. The latter type of conjugate was readily hydrolyzed by the appropriate enzyme, β-glucuronidase. However, the sulfuric esters subjected to Takadiastase were not split quantitatively. Comparative experiments in which enzyme and 0.2 N acid were used led to 25 and 95% of material, respectively, extractable into ether. Chromatography of the ether-soluble fraction gave, nevertheless, the same components, i.e. mainly N-(7-hydroxy-2-fluorenyl)acetamide, and small amounts of the 5-hydroxy derivative. The failure of Takadiastase to split these conjugates could mean that not all of this fraction is composed of sulfates, but consists also of some other materials readily hydrolyzed by acid. Further investigation of this problem might be warranted.

B. Tissue metabolites. Further experiments on the so-called protein binding of radioactivity from carcinogen or carcinogen metabolites to tissue constituents were performed. We and other investigators have reported that labeling of the proteins occurred in animals treated

with radioactive carcinogens. Further experiments showed that this was a complex series of reactions involving a number of tissue constituents and several metabolites of the carcinogen. The problem was to determine which of these reactions was related to the carcinogenic property of the compound. In 1959 we described reactions involving a few selected metabolites of N-2-fluorenylacetamide, namely the aminofluorenols, which gave rise to rat liver protein-bound radioactivity as an artifact. Homogenization of the livers with the compounds gave bound radioactivity at a level as high or higher than that found in in vivo experiments. Now hydrolyses by enzymes of the proteins labeled in vivo or in vitro were performed. In the former case, 50-60% of the radioactivity liberated was extractable into organic solvents, whereas in the latter case no activity was organic-soluble. Thus, the two types of labeling are clearly different. Moreover, the aminofluorenols form only a small part of the metabolites of N-2-fluorenylacetamide, so that it can be concluded that the artifact type of binding plays at best a minor role in the binding noted in in vivo experiments.

As a further point of interest in this connection it was established that homogenization per se was not required in the artifact binding. Thus, mixing of a solution of, for example, 7-amino-2-fluorenol with the supernatant of a centrifuged liver homogenate followed by heat denaturation of the soluble protein, gave rise to high protein-binding of radioactivity. The reaction took place in the presence or absence of oxygen; It was instantaneous and did not increase with time and followed certain stoichometric rules.

Another experiment on the subject of protein-binding of carcinogen was performed for Dr. H. P. Morris, Chief of our Section, with rats bearing the Morris hepatoma No. 5123. This tumor in many respects resembles normal liver. It was of interest, therefore, that the proteins of tumors from subcutaneously or intraperitoneally inoculated rats given a dose of labeled N-2-fluorenylacetamide showed only about 10% of the activity of the proteins of normal liver.

As a preliminary step to further studies on protein-binding of isotope from N-2-fluorenylacetamide, the carbon-14 bound to liver homogenized

at pH 5 and pH 7.8 was determined. In all our previous experiments pH 5 was used for technical reasons. However, some other investigators employed pH 7.8 which is closer to the physiological pH. At the lower pH less soluble protein with a slightly higher specific activity was found than at the higher pH. The reverse was true for the insoluble proteins. The total amount of isotope in the soluble proteins was higher at pH 7.8.

2. Metabolism of N-3-Fluorenylacetamide in Rats.
Our previous work demonstrated that monohydroxylation was an important biochemical reaction undergone by compounds such as N-2- and N-3-fluorenylacetamide (cf. Adv. Cancer Research 5, 331, 1958). A number of unknown, more polar metabolites were suspected to be the dihydroxy derivatives. In order to ascertain the actual presence of this type of compound in the urine of rats studies were performed with the 2,7-dihydroxy derivative of N-3-fluorenylacetamide. The urine of rats injected with this compound was partitioned between solvent pairs, treated with specific enzymes such as β-glucuronidase and Takadiastase, hydrolyzed with acid, and certain of the extracts were chromatographed on alumina, as described under I. 1. A, above. In addition color tests for the compound were developed. The results of all these studies showed that the diol was, indeed a metabolite of N-3-fluorenylacetamide. In addition, another unknown spot on the chromatograms could be identified as N-(7-hydroxy-3-fluorenyl)-acetamide which formed a sizable portion of the urinary metabolites; it was the only one excreted as a sulfuric acid conjugate. The 2,7-dihydroxy derivative was noted mainly as a glucuronide, as was also the previously known 2-hydroxy derivative (cf. J. Am. Chem. Soc. 77: 1914, 1955). Thus, at least in this model system polyhydroxylation does occur, and it is conceivable that some of the as yet unknown materials in the urine of rats dosed with N-2-fluorenylacetamide are of the diol type. Their general properties would not be in disagreement with this concept, and it may be worthwhile to investigate this problem further. The polyhydroxylated compounds possess a number of charged reactive groups which may be responsible for their biochemical and possibly biological properties.

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3. Metabolism of Fluorene in Guinea Pigs and Rabbits.
As noted earlier a number of highly polar, preferentially water-soluble metabolites were encountered in our studies of the fate of N-2-fluorenylacetamide. In order to gain insight into the type of structure responsible for such unusual properties in this series of compounds, it was deemed of interest to use fluorene itself as a model. With fluorene-9-C^{14} it was found that about 10-20% of the urinary radioactivity in rats, guinea pigs, and rabbits was in the form of water-soluble metabolites that were not sulfates or glucuronides. Investigation of their identity is presently underway, and it is hoped that these fundamental studies will throw light on similar derivatives of thefluorenylacetamides.

In connection with these experiments, the urinary metabolites of fluorene were determined. In addition to the known 2-fluorenol and its glucuronide (Biochem. J. 43:533, 1948), another important metabolite in guinea pigs and rabbits was 9-fluorenol, excreted mainly as the glucuronide. In contrast, sulfuric acid conjugates were found only for 2-fluorenol. Evidence for the excretion of another new metabolite, 2,9-fluorenediol, was obtained in preliminary experiments, but further work is required to establish the point with certainty.

The C-14-labeled fluorene also permitted a study of protein-binding of activity to liver proteins. Fluorene is not carcinogenic. However, in rats binding was observed to an extent about 1/2 that observed with carcinogenic N-2-fluorenylacetamide. In rabbits, the level at 48 hrs. was about 1/3 that in rats, and in guinea pigs about 1/5 that of rats. Since difficulties were encountered in the important problem of identifying the metabolites of fluorenylacetamide bound to proteins, the proteins labeled by fluorene may be good model systems with which to gain insight into the structure of the materials bound to protein.

III. Organic-chemical Aspects of Problem

1. Ortho-Substituted Derivatives of N-2-Fluorenylacetamide.
In connection with the development of procedures for the synthesis of derivatives of N-2-fluorenylacetamide substituted in the ortho positions

by the halogen fluorine (cf. reports for 1958 and 1959), some alternate pathways were investigated in the hope of improving theyields of the desired products. Thus, two new routes to the intermediate 3-fluorofluorene were found, in addition to the one used previously. Both serve equally well, but no better than the older mode of synthesis. In this connection a contribution was made to the understanding of the chemistry of ortho-substituted benzophenones; some of these compounds exist and react preferentially in the normal form, others in a pseudo form. Details are in press (cf. ref. 6).

Further attempts at the synthesis of fluorine-containing derivatives of N-2-fluorenylacetamide were based on the introduction of the halogen atom by exchange with another one, for example bromine. The intermediates required were prepared, in part by newly developed methods. However, all the experiments failed to give the expected fluorine derivatives. Instead, the unexpected discovery was made that hydroxy and amino groups could be introduced into the fluorene ring in this manner. Thus, a number of new and known hydroxylated and animated derivatives of 2-fluorenamine were prepared in this fashion. One of these, 2-amino-3-fluorenol, is an important metabolite of the carcinogen in rats, which can thus be prepared readily in good yield; previous syntheses of this compound were considerably more involved. A paper summarizing these studies is in press (cf. ref. 7).

2. Hydroxylated derivatives of N-3-fluorenylacetamide. Last year we reported the successful synthesis of N-(2,7-dihydroxy-3-fluorenyl)-acetamide, which (see part I, 2 of this report) could be shown to be a metabolite of N-3-fluorenylacetamide. In addition, chromatograms of urine extracts of rats treated with this compound showed a spot which a series of tests indicated as possibly due to N-(7-hydroxy-3-fluorenyl)acetamide. A synthesis of this compound was developed to ascertain this point. Starting with N-(7-methoxy-2-fluorenyl)acetamide (J. Chem. Soc. 1954, 758) by a series of reactions involving nitration, separation of isomers, deacetylation, deamination, dealkylation, reduction, and acetylation, the desired compound was obtained. The synthetic product and the metabolite were identical.

3. Reactions of 4,5-methylenephenanthrene. The nitration of this rare hydrocarbon was studied under a variety of conditions, all of which gave a mixture of isomers which remain to be separated. Oxidation of the methylene group in a selective manner gave the ketone; the nitration of this material will also be investigated.

4. Substrates for histochemical studies (with Dr. M. Burstone). Our experience in the chemistry of aromatic compounds, especially in regard to the amino derivatives, has facilitated this collaborative study. As possible histochemical reagents for aminopeptidases, and other enzymes, alanyl derivatives of 12 amines were made. Two of these, derived from 3-aminocarbazole appear quite promising. Details are in press (cf. refs. 3 and 4).

5. New metabolite of N-2-fluorenylacetamide. Our familiarity with the properties of the various metabolites suggested to us that an unknown metabolite reported by Cramer, Miller, and Miller (Proc. Am. Assn. Cancer Res. 3:13, 1959) might be the N-hydroxy derivative. A synthesis based on the partial reduction of 2-nitrofluorene was developed and chromatographic studies indicated that this compound, indeed, corresponded to the unknown of the Millers. At that time we learned that the comprehensive studies of the Millers had reached the same conclusion, although by a different route (cf. Cancer Res. 20: 950, 1960). Hence we abandoned further efforts on this point.

6. Compounds required for the physical-chemical studies, described below, were synthesized and/or purified.

7. Studies on the relationship of ultraviolet and infrared spectra to the chemical structure of the compounds prepared by us were continued. The results were most helpful in proving and in determining the structure of some of the materials.

8. New metabolite of fluorene. Studies on the metabolism of fluorene (cf. I, 3) indicated the possibility that 2, 9-fluorenediol could be a metabolite. This compound was synthesized starting with fluorene by nitration, oxidation, reduction, introduction of hydroxyl, reduction of keto group.

9. Help to other investigators. Samples of compounds synthesized by us were supplied to the following investigators as our contribution to their studies: Dr. Irving (Veterans Administration Hospital, Memphis) Dr. Lipkin (Bellevue Hospital, New York), Drs. Miller (Univ. Wisconsin) Dr. Sorof (Inst. Cancer Research, Philadelphia), Dr. Szent-Gyorgyi

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(Inst. Muscle Research, Woods Hole). In addition 45 g. of N-2-fluorenylphthalamic acid was especially prepared for Dr. Harold P. Morris, of this Section.

III. Physical-chemical Aspects of Problem.

1. Ionization constants of polynuclear aromatic amines and related compounds. Details of this study have been described in 1958 and 1959. While preparing this material for publication the need for certain additional constants arose in order to have enough data on which to base correct theoretical interpretations. Some of these compounds were the dialkyl derivatives of the amines. This problem has now been satisfactorily completed and a paper is in press (ref. 2).

2. Polynuclear aromatic aminophenols as metal chelating agents. Essential details of this problem have likewise been discussed last year. This year we showed by the isolation of metal complexes that the new and important metabolite of N-2-fluorenylacetamide, the N-hydroxy derivative, forms metal chelates. This material is being prepared for publication.

Significance: Our research aims to develop an understanding of the intimate processes surrounding the initiation of the neoplastic processes. This goal is of fundamental importance to cancer research.

Proposed Course of Project: Our future endeavors will follow up the leads discovered in the course of the present work. Some of these are pointed out in the body of this report.

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Weisburger, J. H., and Schneider, W. C.: Improved Preparation of Phosphorylethanolamine and Phosphorylcholine. J. Org. Chem., in press.

2. Grantham, P. H., Weisburger, E. K., and Weisburger, J. H.: Ionization Constants of Derivatives of Fluorene and Other Polycyclic Compounds. J. Org. Chem., in press.

3. Burstone, M. S., and Weisburger, E. K.: New diazonium components as coupling agents in the demonstration of phosphatases. J. Histochem. and Cytochem., in press.

4. Burstone, M. S., and Weisburger, E. K.: Development of new histochemical substrates and diazonium salts for the demonstration of aminopeptidase. ibid., in press.

5. Suzuki, K., Weisburger, E. K., and Weisburger, J. H.: Nitration of 1- and 3-Fluorofluorene. J. Org. Chem. 24:1511, 1959 (in press last year).

6. Suzuki, K., Weisburger, E. K., and Weisburger, J. H.: Derivatives of 3-Fluorofluorene by the Pschorr Synthesis. J. Org. Chem., in press.

7. Suzuki, K., Weisburger, E. K., and Weisburger, J. H.: Replacement and Elimination of Bromine in Bromonitrofluorenones. The Preparation of 2,3- and 1,2,3-Substituted Fluorenes and Fluorenones. J. Org. Chem., in press.

Honors:
Mr. P. H. Grantham was given a Superior Performance Award by NIH during the current year.
Dr. J. H. Weisburger was nominated for service on the Editorial Boards of the JOURNAL OF THE NATIONAL CANCER INSTITUTE and of CANCER RESEARCH.

Serial No. NCI-346 and 349
1. Lab. of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Maryland

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Part A.

Project Title: Influence of Liver Function in Rats Receiving a Chemical Carcinogen and the Variations of Vitamin B_6 Intake as Related to the Development of Liver Neoplasms.

Principal Investigator: H. P. Morris

Other Investigator: B. P. Wagner

Cooperating Units: H. Sidransky, Laboratory of Pathology, NCI

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: Detection of liver changes produced by N-2-fluorenyl-acetamide (2-FAA) as affected by different levels of vitamin B_6 intake.

Methods: Rats of both sexes were fed diets containing high and low levels of vitamin B_6 and 2-FAA. The gross and histological changes were noted over periods of one week to several months.

Major Findings: Male rats that ingested a diet containing 2-FAA and high B_6 had a higher incidence of more anaplastic liver tumors than female rats, however, if mammary tumors were removed surgically from the females they survived longer and developed more liver tumors. Studies of the precancerous period revealed that histological changes occurred sooner and were more extensive in male rats than in females.

Part B included: NO

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Testosterone promoted early histological changes induced by 2-FAA in the livers of male rats compared to intact males.

Rats of both sexes on a diet high in B6 and containing 2-FAA developed tumors earlier than those on a low pyridoxine carcinogen diet. The incidence of liver tumors was not significantly different at either level of pyridoxine but more mammary tumors developed earlier on high levels of B_6 and 2-FAA. Where there was a long survival period more ear duct tumors were found.

Significance: Factors which promote differences in liver and mammary tumor incidence provide differences which may be used to study possible differences in metabolism of the carcinogen.

Proposed Course: Present results are being analyzed and written up. One manuscript was completed and accepted for publication about January 1961 and a second paper is nearly ready for submission for publication.

Serial No. NCI-347
1. Lab. of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Maryland

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Part A.

Project Title: Role of Tryptophan and Indole in the Induction of Bladder Tumors in Rats Ingesting N-2-Fluorenylacetamide (2-FAA).

Principal Investigator: H. P. Morris

Other Investigators: B. P. Wagner, H. M. Dyer

Cooperating Units: H. Sidransky, Laboratory of Pathology, NCI

Man Years (calendar year 1960):
Total: 1-1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To determine the role of tryptophan and indole in the induction of bladder tumors in the rat after ingestion of N-2-fluorenylacetamide (2-FAA).

Methods: Rats of the Fischer strain are on diets containing 2-FAA and modified as to amount of vitamin B_6 and propylthiouracil.

Results: Bladder tumors develop in female Fischer strain rats fed diets low in B6 with or without added DL-tryptophan and containing 2-FAA. Liver tumors also develop in these rats. Three transplantable liver tumors were started from rats on some of the above regimens during the year. A high mortality has occurred in the rats and since bladder tumors develop in females after about 8 or more months on the 2-FAA diets this may adversely affect the results.

Part B included: NO

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Male rats without added tryptophan but containing 2-FAA have not been found to develop liver tumors.

Significance: This project represents another study into the causative factors of carcinogenesis.

Proposed Course: Continuation of animal experiments with the Fischer strain rats in attempts to verify previous preliminary findings.

Serial No. NCI-400
1. Laboratory of Biology
2. Office of the Chief
3. Bethesda, Md.

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Part A

Project Title: Biological studies on the factors involved in the development and growth of tumors in experimental animals.

Principal Investigator: H. B. Andervont

Other Investigators: Dr. Thelma B. Dunn (NCI-LP)

Cooperating Units: None

Man Years (calendar year 1960):
Total: 5
Professional: 1
Other: 4

Project Description: Disappearance of the Mammary Tumor Agent

Breeding Experiments

This report is a continuation of last year's report because the results include most of the known factors involved in the occurrence of mammary tumors of mice.

The study was started to elucidate the reasons why the agent disappeared suddenly from a few strain RIII mice; tumors did not occur in these mice or in their descendants. Two possibilities may be responsible. First, the RIII mice carry an agent of low activity. Second, the genetic

constitution of certain mice makes them resistant to the agent.

The first possibility was easy to explore by the acquisition of reciprocal F_1 hybrids derived from strain RIII and strain C3H. The latter is a high tumor strain and should carry an agent of high activity. To determine the relative potencies of the agents, the F_1 hybrids were procured. Each F_1 hybrid female bore one litter which was killed soon after birth. It was known that (RIII X C3H)F_1 hybrids developed tumors in the absence of the agent when exposed to strong estrogenic stimulation, and this called for the use of control mice procured by mating presumably agent-free females of one strain to males of the other strain.

The experiment was not limited to female F_1 hybrids. Each F_1 hybrid male, when 2 months old received, subcutaneously, a pellet containing 10 per cent diethylstilbestrol dissolved in cholesterol. These mice should also reflect the activity of the agents and serve as controls for their female litter mates.

The experiment is completed, and all tumors have been diagnosed histologically. The findings are shown in table 1 which includes all mice. The results in F_1 hybrids born to mothers and fathers that carried the agent showed that the C3H agent is more potent than the RIII agent. The average age at which tumors arose in (C3H+ X RIII+)F_1 hybrids was lower than the average age for tumor development in the reciprocal (RIII+ X C3H+)F_1 hybrids. This finding was similar to the results published from another laboratory while our experiment was in progress and can, therefore, be accepted without further confirmation. It showed that the relatively weak agent carried by RIII females could be a factor involved in the disappearance of the agent from an occasional mouse.

Table 1. - Occurrence of mammary tumors in breeding females of strains RIII and C3H and in their reciprocal hybrids.

Derivation *	Sex	No. of mice	No. with tumors	Incidence (percent)	Average age (months)	No. dead without tumors	Average age (months)
RIII+	F	23	22	96	12.7	1	20
C3H+	F	23	16	70	10.2	7	10.3
RIII-	F	26	1	4	12	25	21.6
C3H-	F	10	0	0	-	10	21.8
RIII+ X C3H+	F	62	59	95	13.4	3	24
" X "	M	56	37	66	12.5	19	22.4
C3H+ X RIII+	F	63	63	100	7.7	0	-
" X "	M	56	43	77	9.7	13	22.8
RIII- X C3H+	F	76	28	37	19.6	48	23.1
" X "	M	70	30	43	14.8	40	22.7
C3H- X RIII+	F	33	5	15	16.4	28	22.7
" X "	M	30	4	13	13.7	26	22.8

*A + sign denotes the presence of the mammary tumor agent; a - sign denotes its absence.

An interesting and unexpected finding was the higher incidence of tumors in F_1 hybrids derived from agent-free (RIII-) females and C3H+ males than in the reciprocal hybrids procured from C3H- females and RIII+ males. Two factors could be involved. First, the genetic makeup of the F_1 hybrids. Our agent-free line of C3H mice has been separated from the agent-carrying colony for 40 generations. Thus, the use of C3H+ males and C3H- females could account for the difference in response of the F_1 hybrids to the same amount of hormonal stimulation. Second, the agent, or a variant of the agent, could be persisting in the RIII- females and not evoking tumors in them.

The problem can be attacked. Each RIII- female can give birth to 2 litters; one born to a C3H+ father and one to a C3H- father. Also, each C3H- female can bear 2 litters; one to a RIII+ and one to a RIII- father. If all F_1 hybrids derived from C3H+ mice show higher incidences of tumors than those procured from C3H- mice, then the difference between them could be attributed to a substrain difference between the colonies of C3H mice.

The experiment could be extended to include F_1 hybrids born to RIII+ females bred to C3H+ and C3H- fathers and to C3H+ females bred to RIII+ and RIII- fathers. This would produce 8 groups of hybrids which could be used to detect viruses as outlined by Dr. Bryan in project report NCI-420 (f). Four would serve as controls known to contain the virus and 4 unknowns which may or may not harbor the virus. Detection of the virus in offspring born to RIII- mothers and C3H- fathers would show that the RIII- mothers were, indeed, carrying the virus. The proposed study would give Dr. Bryan and his colleagues the advantage of knowing which virus they were seeking as well as the possibility of revealing another virus as an etiological factor in the occurrence of breast tumors of mice.

Foster Nursing Experiments

Another approach to the disappearance of the agent from RIII mice was to introduce the RIII agent into agent-free C3H mice and the C3H agent into agent-free RIII mice. This was done by foster nursing. The fostered mice were mated to their brothers to obtain the F_1 generation and all later generations were obtained by brother to sister matings. Only the fostered mice received milk from the other strain.

The purpose of these efforts was to ascertain whether the weak RIII agent would disappear from the C3H strain females and whether the more potent C3H agent would disappear from the RIII females. If the latter occurred, then the RIII genetic makeup could also be responsible for the agent's disappearance.

The results thus far of exposing strain C3H to the RIII agent are shown in table 2. The mice were derived from 11 strain C3H females foster nursed by RIII mice. Each fostered mouse was the ancestor of a family. In the first 3 families 5 generations are completed and in the last 8 families 3 generations are completed. The latter families were established after 3 generations of the first 3 families had shown that the RIII agent was transmitted through subsequent generations of C3H females.

Table 2.--Occurrence of mammary tumors in strain C3H mice after acquiring the RIII mammary tumor agent

Generation	Families 1-3						Families 4-11					
	No. of mice	No. with tumors	Tumor incidence (percent)	Mean age (months)	Mean age at death without tumors (months)	Mean age of living mice (months)	No. of mice	No. with tumors	Tumor incidence (percent)	Mean age (months)	Mean age at death without tumors (months)	Mean age of living mice (months)
Fostered	3	1	33	19	18.5	-	8	3	37	15	21.6	-
F_1	7	5	71	15.8	22.5	-	26	16	61	16.1	18.1	-
F_2	19	4	21	16.2	21.7	-	43	12	28	14.7	17.3	-
F_3	41	9	22	18.9	20.6	-	61	20	33	15.2	13.7	22
F_4	33	7	21	17.9	20.8	-						
F_5	22	0	-	-	16.7							
F_6	37	5	13	18.4	17.6	22						

The findings, thus far, confirm the lower activity of the RIII agent in C3H mice. When exposed to their own agent breeding C3H females show a tumor incidence of 95 per cent at a mean age of 8.5 months. All mice included in table 2 bore one litter; some nursed their litters to procure the next generation, but most were removed from their young within 24 hours after delivery.

Details of the tumor history of each family are not included here. Thus far, there is no unequivocal indication that the RIII agent has disappeared from the C3H mice but evidence is accumulating that there is considerable variation between the tumor incidences of different families. For example, in family 2 no tumor has appeared in any member beyond the F_3 generation. If this record is maintained, it will suggest that the RIII agent has disappeared from mice of another strain.

More interest is attached to the disappearance of the potent C3H agent from strain RIII mice. Only 4 families were started from 4 RIII females suckled by C3H females, because strain RIII mice are very difficult to maintain. The families are now in the F_9 generation of inbreeding and are showing a striking difference in tumor incidence. Table 3 summarizes the first 8 generations of family 2. The incidence of tumors is 100 per cent in 6 of 7 completed generations, and the mean tumor age is remarkably low. Attention is directed to a mouse of the F_2 generation that lived for 25 months and died tumor free. This animal shows that, even within a highly susceptible family, an occasional RIII mouse is very resistant even to the potent strain C3H agent. Two sisters developed tumors when 5 and 7 months old. Unfortunately, the resistant mouse failed to raise the 3 litters she bore.

Table 4 includes the findings in family 3, the members of which, from the first, have been more resistant to the development of tumors. From the F_2 generation on, some of these animals lived their normal life spans without developing tumors. Thus far the following line within the family shows good evidence that the agent disappeared from a mouse of the F_4 generation.

Table 3. - Family 2. Occurrence of mammary tumors in strain RIII mice after acquiring the strain C3H agent

Generation	No. of mice	No. developed tumors	Mean age (months)	Mean age at death without tumors (months)	No. living	Mean age (months)
F	1	1	5	-	0	-
F_1	3	3	7.5	-	0	-
F_2	3	2	6	25	0	-
F_3	7	7	8.5	-	0	-
F_4	10	10	8.4	-	0	-
F_5	6	6	7.7	-	0	
F_6	6	6	6.2	-	0	-
F_7	7	6	8.1	6	0	
F_8	11	10	5.4	-	1	7.5

Table 4. - Family 3. Occurrence of mammary tumors in strain RIII mice after acquiring the strain C3H agent

Generation	No. of mice	No. developed tumors	Mean age (months)	Mean age at death without tumors (months)	No. living	Mean age (months)
F	1	1	13	-	0	-
F_1	4	3	14	12	0	-
F_2	7	6	9.7	26	0	-
F_3	11	9	12.7	26	0	-
F_4	9	7	9.4	24	1	25.5
F_5	9	7	8.3	15	1	20.5
F_6	3	0	-	-	3	16
F_7	8	0	-	-	8	12.7
F_8	17	0			17	7.1

F_4 Gen. Mouse No. 123 living at 26 months.

F_5 " " " 159 died tumor free at 15 months.

F_6 " " " 191 living at 18 months.

F_7 " 4 mice --- living at 14 months.

F_8 " 10 " --- living at 7 months.

Of 17 mice in the above generations, none has, as yet, developed a tumor.

The results in family 4 suggest that the C3H agent can also disappear from RIII mice. Thus, the importance of the host in the disappearance of the agent is again emphasized.

Part B included Yes X No

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Andervont, H. B., Shimkin, M. B., and Canter, H. Y.: Susceptibility of seven inbred strains and the F_1 hybrids to estrogen-induced testicular tumors, and occurrence of spontaneous testicular tumors in strain BALB/c mice. J. Nat. Cancer Inst. (in press).

Andervont, H. B., Shimkin, M. B., and Canter, H. Y.: Some factors involved in the induction or growth of testicular tumors in BALB/c mice. J. Nat. Cancer Inst. (in press).

Honors and Awards relating to this project: None

Serial No. NCI-433
1. Laboratory of Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies on the viral etiology of neoplasms.

Principal Investigator: Sarah E. Stewart

Other Investigators: Drs. Cecilie and Rudolf Leuchtenberger, Childrens Hospital, Boston; Dr. Robert Cramer, Institute Cancer Research, Roussy, Villejuif, France; Dr. Noriaki Ida, M. D. Anderson Hospital, Houston, Texas.

Cooperating Units: None

Man Years (calendar year 1960):
Total: 6
Professional: 2
Other: 4

Project Description:

A. Work on polyoma virus.

1. A study of the cytological, histological and cytochemical effect of polyoma virus on epithelial and stromal cells of mouse and hamster kidney was carried out as a collaborative study with Drs. Cecilie and Rudolf Leuchtenberger. The work confirmed and extended previous observations showing that there are two main processes in the tissue cells of the kidney which are strikingly different from each other: A degenerative process in the epithelial cells associated with viral activity leading to destruction of cells and a proliferative process in the stroma without evidence of viral activity but associated with cellular activity leading to further propagation of abnormal cells resulting in tumors. The viral activity in the epithelial cells preceded the proliferating activity of the stroma cells. There were only minor changes in the DNA content of the affected

epithelial cells, while the DNA content in the proliferating stroma cells was increased in accordance with DNA synthesis and mitosis.

It was indicated in this study that viral proliferation occurred only in the epithelial cells and that only the stromal cells became malignant. This is in accord with our previous observations as no renal carcinomas have been found in mice injected with polyoma virus but renal sarcomas are common. Proliferation of tubular epithelium, however, was frequently observed. The observation which evolved from the cytochemical studies is that a cell responds in either one or the other manner to viral infection, suggesting the development of 2 different virus-host-cell relationships. In the one instance the cell is used by the virus for its replication suppressing cell mitosis and finally killing the cell, while in the other instance the virus or its products acts as a stimulating agent provoking reduplication of cells resulting in malignancy. This study was made in collaboration with Drs. Cecilie and Rudolf Leuchtenberger.

2. Studies on zone electrophoresis on the hemagglutinin of hemagglutinating and of a masked polyoma virus were carried out in collaboration with Dr. Robert Cramer.

The studies were made to test the possibility that a definite virus-inhibitor dissociation could be achieved in zone electrophoresis. The masked strain with an HA titer of 1:2 - 1:4 was found to increase to 1:8 after 1 month's storage at -70° C. After heating at 56° C for 60 minutes the HA titer increased to 1:64. On storage at 4° C for 24 hours masking occurred again. By electrophoresis it was possible to remove a fraction so that the same HA titer of 1:32 was observed whether the fraction was tested immediately after completion of electrophoresis or after having been stored for 24 hours at 4° C. Saccarose in the environment of the electrophoresis column was ruled out as a contributing factor.

Studies are in progress on the electrophoretic mobility of the tumor inducing capacity of the virus.

3. Placental transmission of polyoma virus.

This study is in progress in collaboration with Dr. Noriaki Ida.

Pregnant (NIH White) Swiss mice ranging from 1 to 18 days gestation were inoculated subcutaneously with polyoma virus. The fetuses were taken at term by caesarean section and were foster nursed by non-infected mothers. Of a total of 63 injected pregnant mice 46 had litters which ranged in numbers from 3 to 10. None of the offspring of the inoculated mothers developed tumors in a

12-month observation period at which time 163 were still living. Seventy-eight of the 163 were tested for antibody to polyoma virus and were found to have titers ranging from 1/640 to 1/20,480, indicating the "in utero" passage of the virus. Exposure to the virus after birth, however, cannot be ruled out as the mice had to be housed in a polyoma virus infected room.

Of the 163 survivors 89 were irradiated giving 150 r for 4 times at 3-day intervals. The irradiated and non-irradiated are being held for observation.

A similar study is in progress on the placental transmission of polyoma virus in hamsters.

4. Viral studies on transplanted polyoma virus induced hamster tumors.

Eight tumors which had been carried as tumor grafts for one year or longer were checked for the presence of polyoma virus by culturing in mouse embryo cell cultures. The cultures were carried for 4 passages. They remained free of virus specific cytopathogenic changes and free of hemagglutinins. Virus release could not be demonstrated by subjecting the cultures to radiation or to different temperature exposures. In a 6-month observation period mice inoculated with the cultures have remained free of tumors.

B. Work with human neoplasms.

1. It was earlier reported that certain cell-free concentrates from human neoplastic material produced proliferative pocks in primary cultures of human embryonic cells. The following studies are now in progress to attempt demonstration of the agent responsible for the proliferative effect.

(a) Cultivation of the culture fluids and cells on embryonated eggs of different ages, inoculating the eggs through different routes and examining the chick tissues, the amnion and chorioallantoic membranes both grossly and histologically for evidence of virus activity.

(b) Subjecting the cultures to different doses of radiation and inoculating the fluids from irradiated cultures into fresh primary human amnion cultures and into animals.

2. Studies of lesions in hamsters that were injected when newborn either with fluids from embryonic tissue cultures which had received neoplastic material or with fluids from non-inoculated control cultures.

In a group of 160 hamsters over 12 months old which received fluids from the inoculated cultures the following tumors and lesions were found: (Many animals had more than one primary tumor and other lesions), 18% plasma cell tumors and reticulum cell sarcomas, 6% leukemias, 10% adrenal tumors, 20% liver cysts, many of which were cholangiomas and hemangiomas, 20% spleen atrophy, 5% spleen hemangiomas, 30% acute enteritis, 20% hyperplasia of the mucosal glands of the gut, 20% polyps of the bowel and 14% bowel carcinomas, stomach lesions consisting of 8% ulcers, 2.5% adenomatosis and 8% with cellular changes at the junction of the squamous epithelium with the columnar epithelium; 9% had an epidermoid carcinoma of the esophagus and 7% of the females developed a trophoblast lesion of the uterus. The uterine lesion was more common in the younger age group, 45% of 87 females less than 12 months old had this lesion.

The animals which received the control culture fluids developed essentially the same tumors with the same incidence.

The possibility was considered that the uterine lesion and the high incidence of neoplasms resulted from an induced tolerance to embryonic cells brought about by the tissue culture inoculum. Results obtained in mice which were inoculated for another purpose appear to substantiate this observation; 56% of 276 Swiss mice over 12 months old which had been inoculated when newborn with culture fluids from control mouse embryo cells developed tumors as follows: 20% lung, 15% leukemia, 14% mammary, 6% uterine and 3% other types. In 103 litter mates that received culture fluids plus rabbit antisera (containing antibodies to mouse embryo cells) no unusual tumors were seen, and only 4 mammary and 1 uterine tumors were found. The incidence of lung tumors 18% and leukemias 14% was essentially the same in the 2 groups. These 2 tumors are prevalent in this mouse strain. Mammary tumors (5%) and uterine tumors 0.5% were also found in a group of 61 mice 12 months old or less that did not receive serum, and were not seen in mice of the same age group that had received serum.

Part B Included Yes X No

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Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Stewart, S. E.: Neoplastic Disease (Tumor-Virus Relations). Annual Review of Medicine, Palo Alto, California Annual Reviews, Inc. 1960, pp. 15.

Stewart, S. E., Eddy, B.E., and Stanton, M. F.: Progress in Virus Research. The Polyoma Virus Experimental Tumor Research. Basel, Switzerland, S. Karger, 1960. Vol. 1, p. 67-85.

Stewart, S. E.: Polyoma Virus, Scientific American, 203: 63-71, 1960.

Cramer, R. C., and Stewart, S. E.: Zone Electrophoresis Studies on Hemagglutinin of Hemagglutinating and of Masked Strain of Polyoma Virus. Proc. Soc. Exper. Biol. Med. 103: 697-700, 1960.

Stewart, S. E., and Irwin, M.: Possible Complications in the Use of Laboratory Animals for Studies on the Viral Etiology of Human Neoplasms. Proc. IV National Cancer Conference. (In press).

Serial No. NCI-404

1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Cellular mechanisms in the control of DNA synthesis: Synthesis of deoxynucleoside triphosphate by deoxynucleotide kinases of normal and tumor tissues.

Principal Investigator: Ruth K. Kielley

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1
Professional: 1
Other: 0

Project Description:[1]

Objectives: This investigation is part of a program in which the purpose is to further our understanding of how cell growth and multiplication are controlled by enzyme activities in DNA synthesis. The objectives of the present study

[1] Abbreviations: DNA = deoxynucleic acid; ATP = adenosine triphosphate; TMP = thymidylic acid; d-CMP = deoxycytidylic acid; d-GMP = deoxyguanylic acid; d-AMP = deoxyadenylic acid; d-CTP = deoxycytidine triphosphate; d-GTP= deoxyguanosine triphosphate.

were to characterize some of the conditions influencing deoxynucleotide kinase activities in liver homogenates and to determine the extent to which these enzymes can synthesize deoxynucleoside triphosphates, the direct precursor substances of DNA, in normal mature cells.

Methods Employed: Deoxynucleotide kinase activity was measured by the assay method of Kornberg and associates. P^{32}-labeled deoxynucleotides prepared from labeled DNA (E. coli) are phosphorylated in the presence of ATP to the di- and triphosphates by kinase enzymes in the tissue being assayed. The amount of conversion to the polyphosphate form is determined by treating the enzyme digest with a phosphatase that specifically destroys unreacted deoxynucleoside monophosphate; then adsorbing the phosphatase-resistent nucleotides onto norite and finally, counting the radioactivity of the norite. The radioactivity of the norite fraction is a measure of the amount of deoxynucleoside di- and triphosphate formed from the monophosphate by kinase enzymes of the tissue.

Principal Findings: Various factors affected deoxynucleotide kinase activities in mouse liver homogenates. These were:

1. Composition of the homogenizing medium - The following data illustrate this effect:

Homogenizing Medium	Deoxynucleotide Kinase Activity*		
	TMP	d-CMP	d-AMP
0.05 M glycylglycine, pH 7.0	12	13	96
0.25 M sucrose	12	10	35
Water	9	12	10
0.25 M sucrose -0.025 M glycylglycine - 2 x 10^{-3} M $Cacl_2$-2.5 x 10^{-4} M $Mgcl_2$	4	14	22

* units per gram of liver.

The homogenizing medium of choice was 0.05 M glycylglycine.

2. Substrate concentration - At 6.4×10^{-5} M, the substrate concentration of the standard assay method, maximum rates of synthesis by the kinases of d-CMP, d-GMP and d-AMP were not achieved. At 2.4×10^{-3} M, these kinase activities were increased 10-fold, yet at this high substrate level, the enzymes did not appear to be near saturation. TMP kinase was unique. Nearly maximum rates were obtained at 1.5×10^{-4} M TMP and the rate was only slightly lower at 6.4×10^{-5} M substrate.

3. Enzyme concentration and time course - TMP kinase activity was proportional to enzyme concentration to about 5 mgms. of liver. The time course was linear for 30 to 40 minutes. Maximum conversion of TMP to the di- and triphosphate forms never exceeded 15 per cent. With increasing time of incubation or increasing amounts of enzyme, the radioactive reaction products began to disappear.

The enzyme concentration-activity curves of d-CMP kinase always showed an initial lag phase (up to 4 mgms. of liver) which was followed by a rapid linear rate (to 10 mgms. of liver). At intermediate enzyme concentrations, rates were linear for 30 min. At longer time periods, the rate declined, but incorporation continued. These characteristics suggest that the synthesis of d-CTP from d-CMP involves the participation of two enzymes and that once formed, the products can be maintained.

The kinetics of d-GTP formation indicate that active degradative reactions are superimposed on d-GTP synthesis. The time course was scarcely linear for more than 4 or 5 minutes. Incorporation of radioactivity reached a maximum in about 10 minutes. Thereafter the reaction products began to disappear quickly and after 20 minutes, very little radioactivity remained in the phosphatase-resistant fraction. Enzyme concentration-activity curves of d-GMP kinase were characterized by a lag phase at low enzyme concentrations suggesting again the possibility of two distinct kinases. The linear portion of these curves represented enzyme concentrations between 10 and 15 mgms. of liver.

Linear relationships of d-AMP kinase activity with enzyme concentration and time occurred between 0.02 and 0.05 mgm. of liver and at time periods up to 10 minutes.

Some deoxynucleotide kinase activities of mouse and rat liver are given below. Activities were determined at a substrate concentration of 6.4×10^{-5} M and at enzyme concentrations and time periods where rates were linear.

	Deoxynucleotide Kinase Activity*			
	TMP	d-CMP	d-GMP	d-AMP
Mouse liver	13.2	10.8	10.1	126
	10.6	14.9	8.7	112
	12.0	19.1	9.9	171
	9.3	10.3	12.2	120
Rat liver	2.9	25.9	18.9	77
	2.5	20.3	19.7	71
	3.4	22.6		110

* Units per gram of tissue. A unit is defined as that amount of enzyme that converts 100 millimicromoles of deoxynucleotide to a phosphatase-resistant form in 1 hr. at 37° under the conditions of the assay.

Significance: The information gained from these studies on the conditions and characteristics of formation of deoxynucleoside triphosphates as primary precursor materials of DNA is basic to attempts to find fundamental differences between normal and tumor cells in growth control mechanisms. In normal mouse liver it has been shown that the synthesis and maintenance of each deoxynucleoside triphosphate follows a distinct and characteristic course, conditioned by the activity of the individual deoxynucleotide kinase and by what appear to be selective degradative activities that destroy precursor material. In non-growing mature cells, it is conceivable that activities catalyzing the destruction of precursor substances may play a dominant role in controlling DNA synthesis. The low TMP kinase activity of rat liver is in accord with observations made by others. In mouse liver, TMP kinase was 3 to 5 times more active. It remains to be seen whether this enzyme activity can be considered a limiting factor in DNA synthesis in normal adult liver. From studies of substrate concentration on other deoxynucleotide kinase activities, however, one can conclude that the enzyme levels of d-CMP, d-GMP and d-AMP kinases are comparatively high and would not therefore be limiting factors in DNA synthesis.

Proposed research: Similar studies of deoxynucleotide kinase activities and deoxynucleoside triphosphate synthesis with tumor tissues are now in progress and will be continued. Preliminary results indicate that the course of deoxynucleoside triphosphate synthesis in tumors is radically different in some respects from that found in normal liver. These points of difference will be investigated and related to specific enzyme activities as far as it is possible. It is likely that deoxynucleotidases and deoxynucleoside triphosphatases will be involved in these studies.

Subcellular distribution studies of TMP kinase activity in mouse liver will also be continued. These studies are particularly interesting because of a preliminary finding that a factor in the soluble fraction prevents accumulation of thymidine triphosphate. Attempts will be made to learn the nature of the inhibitory factor.

Part B Included Yes No X

Serial No. NCI-405
1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies on the origin of the deoxyribosides of rat tissue and the metabolism of these compounds during liver regeneration and tumor growth.

Principal Investigator: Jean Rotherham

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: To isolate and identify the deoxynucleosides found in the urine, normal tissues, tumors and regenerating liver tissue of rats; to determine the endogenous origin of these compounds and to investigate the possible relationship between the metabolism of these compounds and of deoxyribonucleic acid (DNA).

Methods: Tissues are removed from anesthetized animals and immediately frozen in liquid nitrogen. Daily 24-hour urine samples are collected in perchloric acid in modified plastic cages which are autoclaved daily to minimize bacterial growth. The acid-soluble deoxyribosyl compounds are separated from acid-insoluble tissue components by making cold perchloric or trichloroacetic acid extracts of the tissues and the urine. The organism, L-acidophilus R-26, which specifically requires deoxyribosyl compounds for growth is

used routinely to determine the content of deoxyribosyl compounds in these extracts and to follow the isolation and purification of these compounds for identification. The compounds were isolated and purified by paper and column chromatography and identified by ultra-violet absorption spectra and by microbiological and chromatographic behavior.

Major Findings:

A. Identification of deoxyribosyl compounds found in rat urine and liver and kidney.

Urine from normal rats which were fed a casein diet was found to contain unidentified deoxynucleotides, deoxycytidine, 5-methyldeoxycytidine and deoxyuridine in the approximate relative amounts of 5, 60, 20 and 20 per cent respectively.

Since, in previous studies on the identification of deoxyribosyl compounds in normal rat liver, regenerating liver and in the Novikoff hepatoma, the chromatographic solvents used would not have separated deoxycytidine from 5-methyldeoxycytidine and the latter may have been present but undetected, these isolation studies on the urine extracts are being repeated with extracts of normal liver and kidney but are incomplete.

B. Studies on the source of urinary deoxyribosyl compounds.

The feces of rats which were fed laboratory chow contained a relatively large amount of deoxyribosyl compounds whereas these were absent from the feces of rats which were maintained on a synthetic diet containing no source of preformed deoxyribosyl compounds. It appeared from this that deoxyribosyl compounds were released from the food in the intestine and might be absorbed into the blood. Deoxyribosyl compounds are continuously excreted in the urine of rats when they are maintained for two or three weeks on a diet which contains no source of preformed deoxyribosyl compounds. This indicates that a considerable part of the deoxyribosides excreted in the urine are endogenous although bacterial action has not yet been eliminated as a source.

C. Studies of the effect of liver regeneration and hepatoma growth on the level of excretion of deoxyribosides in rat urine.

A comparison was made of the levels of urinary excretion of deoxyribosides between rats undergoing liver regeneration and pair-fed controls when both groups were fed laboratory chow. There is considerable variation in these excretion patterns.

However, the preliminary data show that the level of urinary excretion of deoxyribosyl compounds falls in the controls when the food intake is restricted to correspond to the food intake of the hepatectomized animals which is low on the day following partial hepatectomy. For the hepatectomized animals the excretion level does not drop until the second day following liver regeneration. These differences may be related to the synthesis of deoxyribonucleic acid or its precursors which begins and is almost complete within 24 hours following partial hepatectomy.

The excretion of deoxyribosyl compounds in rats following implantation of the Novikoff hepatoma falls to a low level during the period of rapid growth of this tumor just before the death of the animal. However, for control animals which are pair-fed on a chow diet, the excretion level drops similarly.

In order to clarify thses findings it seemed necessary to control more of the variable factors involved. Consequently similar excretion studies were carried out with animals that were fed a synthetic diet which was free of any source of preformed deoxyribosyl compounds, since the laboratory chow is unsatisfactory in this regard. The differences in excretion patterns between the experimental animals and the pair-fed controls were similar to those for animals fed a chow diet in that the level of excretion fell in the experimental animals on the second day after hepatectomy. On the synthetic diet the excretion level was lower for all animals and did not fall in the pair-fed control animals on the day of lowered food intake.

Significance for Cancer Research: A more complete understanding of the over-all metabolism of deoxyribosyl compounds in animals and the relationships between this and the growth of normal and malignant tissues may help to clarify factors related to the metabolism of DNA during growth of tissue. Since the synthesis of DNA is an essential preliminary step in tissue growth, more complete knowledge of the pathways of precursors and products of the metabolism of DNA may yield data of importance to an explanation of some of the biochemical aspects of tumor growth.

Proposed Course of Project: If 5-methyl deoxycytidine is found to be an endogenous metabolite in rat tissues, it will be desirable to study the *in vivo* and *in vitro* formation of this compound with the use of C^{14}-deoxycytidine and other possible labeled precursors.

The studies on urinary excretion of deoxyribosyl compounds during liver regeneration and tumor growth for animals maintained on a synthetic or partially synthetic diet should be continued to obtain more complete data. These should also be extended to normal

germ-free animals fed a partially synthetic diet.

It would be desirable to investigate the metabolism of deoxyribosyl comppunds in the perfused livers of rats using labeled precursors and attempting to identify the products formed.

Part B Included Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Rotherham. J., and Schneider, W. C.: Deoxycytidine, deoxyuridine and 5-methyldeoxycytidine in rat urine. Biochim. Biophys. Acta 41: 344-345, 1960.

Honors and Awards relating to this Project: None

Serial No. NCI-406
1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Ultrastructure of normal and neoplastic cells.

Principal Investigator: Albert J. Dalton

Other Investigators: Margaret D. Barrett, Morris Belkin, W. R. Bryan, W. E. Heston, L. W. Law, Ruth Merwin, J. B. Moloney, Oppelt, M. Potter

Cooperating Units: Leukemia Service. Clinical Center.

Man Years (calendar year 1960):
Total: 3
Professional: 1
Other: 2

Project Description:

Study A. Oncogenic viruses.

Objectives: This study is a logical continuation and extension of that which has been in process since 1950. During this interval attention was first focused on the ultrastructure of normal cells and more recently on neoplastic cells since it was felt that interpretation of results on the latter would be impossible without a broad and basic understanding of the former. Within the last few years it has become apparent that mature forms of a number of viruses including oncogenic ones may be identified by their characteristic ultrastructure. With this knowledge at hand several studies have been initiated with the aim of gathering information concerning the ultrastructure and mode and site of formation of 3 oncogenic viruses of the mouse - those associated with (1) the Friend and (2) the Moloney leukemia,

and (3) that associated with mammary tumors. The hope, of course, is that the information obtained in these studies will be in some way applicable to the study of human neoplasms.

A preliminary study of the first of these. the Friend leukemia, was carried out at the Sloan-Kettering Institute for Cancer Research during a year's leave of absence for study at that Institution. The experience and information from this study became immediately applicable in the study of the Moloney leukemia in collaboration with Drs. Moloney and Oppelt. Samples of the various lymphoid organs - lymph node, spleen, thymus and bone marrow were taken for analysis with the electron microscope as well as samples of subcutaneous transplants of tumor cells and tissue culture cells carrying the virus as demonstrated by bioassay. The development of a stain technique which stains nucleo proteins preferentially has been of particular value in this study (see publications).

Major Findings: The results of the electron microscopic analysis of the Moloney leukemia have indicated that while all lymphoid organs contain mature virus particles in large numbers and while viral particles have been found in the process of budding from the plasma membrane of neoplastic cells, the primary site of formation and apparently the major source of virus are the megakaryocytes of the bone marrow (and to a lesser extent megokaryocytes of the spleen). The majority of particles in any one megakarocyte were in the same stage of development, the stages varying from one cell to the next. In nearly all megakaryocytes the particles were in some stage of development and seldom possessed the ultrastructural features characteristic of the mature particle found in such large numbers extracellularly in the various lymphoid organs. With this information as a lead, platelets from rats rendered leukemic with the Moloney agent were examined. Many platelets were found which contained particles and occasionally platelets were found which contained numerous centrally located particles. Megakaryocytes and platelets of control rats were found to be negative for the particles. The platelets in one case of human lymphoic leukemia previously treated with an antileukemic agent has been examined and was found to be negative.

Significance to bio-medical research and the program of the Institute: It is perhaps too much to expect but the significance of positive findings in cases of human leukemia are obvious.

Proposed Course of Project: It is proposed to study a series of untreated human lymphoid leukemias in the same manner to determine whether or not a comparable situation exists. A study related to this is being carried out in collaboration with Dr. Law. Tissues of AKR and Figge mice with leukemia are to be analyzed with the electron microscope to determine whether or not comparable particles are associated with these leukemias. A filterable agent is suspected in AKR mice while the dominant factor in the development of leukemia in Figge mice is considered to be a genetic one.

The study of the third virus, that associated with mammary tumors of the mouse, may appear to be of academic interest only, but the solution of some of the problems related to this agent may have much wider significance and application. Two morphologic entities have been found in association with mammary tumor cells. One particle (type A) has always been found in an intracellular position while the other (type B) has been found budding from the plasma membrane or lying free in the acinar lumena. Both types of particles have been found in association with the cells of hyperplastic nodules and with neoplastic cells. Both have also been found in animals presumably free of the mammary tumor agent as determined by bioassay. Electron microscopic studies on this agent have in the past been restricted to one strain. In collaboration with Drs. Heston and Margaret Barrett a long-term study has been developed which will analyze, in a series of strains, mammary tumors appearing in mice with and without the mammary tumor agent. In addition, mammary tumors arising in a resistant strain following introduction of the mammary tumor agent will be analyzed. The aim of this study will be to determine whether or not the particles (types A & B) are (1) strain specific and (2) are primarily related to the development of hyperplastic nodules or mammary cancer or both.

Study B.

In collaboration with Drs. Potter and Merwin a study is in progress involving the analysis of plasma cell tumors with the electron microscope to determine whether or not function as indicated by the elaboration of specific myeloma globulins could be correlated with variations in the ultrastructural features of the neoplastic plasma cells. Results have been obtained on a series of 17 primary and transplanted plasma cell tumors and controls consisting of normal stimulated and unstimulated plasma cell tumors or fibrosarcomas. These results indicate that maintenance of ultrastructural features characteristic of plasma cells is related to retention of function. The evidence also suggests that particles present in the cytoplasm of cells of all of the plasma cell neoplasias examined (but not in normal plasma cells, nor in the cells of the other neoplasias) are not the morphologic representation of an oncogenic agent responsible for the development of the tumors. In an extension of this study it is planned

to study the process of the development of plasma cell tumors in an attempt to determine (1) the cell of origin and (2) the stage in the process at which the particles make their appearance.

Study C.

In collaboration with Dr. W. R. Bryan a study in progress for several years involves the counting of viral particles associated with cells of the Rous sarcoma to determine the relationship between the number of particles, potency of extract used, latent period and potency of extract obtained from the tumor on which the counts were made. A preliminary report showing that a relationship does exist has been published but a more detailed study involving a wider range of doses is in process.

Study D.

In collaboration with Dr. W. R. Bryan pellets obtained from homogenization and differential centrifugation of human tumors are to be examined to determine whether or not virus-like particles are present. Positive findings on any of this material will be followed by bioassay using tissue culture and newborn mice to determine the presence or absence of virus.

Study E.

In collaboration with Dr. Belkin a study has been carried out which has been concerned with determining the site of origin of the vacuoles which are induced to form in the cells of a series of ascites tumors. In one case the vacuoles were found to arise at the site of virus formation (S-37). In a second case vacuoles developed from inclusion bodies in the cytoplasm (Ehrlich ascites tumor). In a third case macroscopic vacuoles arose from pre-existing ultramicroscopic vacuoles (Yoshida ascites tumor).

Part B Included Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Dalton, A. J.: A simplified method of staining thin sections of biological material with lead hydroxide for electron microscopy. J. Biophys. Biochem. Cytol. 7: 409, 1960.

Dalton, A. J.: Morphology and physiology of the Golgi complex. XIVth Annual Symposium on Fundamental Cancer Research, 1960.

Honors and Awards relating to this project:

Elected director, Electron Microscope Society of America.

Appointed to the Council, A.A.A.S.

Serial No. NCI-407
1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Deoxyribose-containing compounds in normal, regenerating, and cancer tissues.

Principal Investigator: Walter C. Schneider

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: 1) To isolate and identify the deoxyribonucleosides and deoxyribonucleotides present in normal and cancer tissues and in body fluids.

2) To determine the metabolic pathways in which these compounds are involved and their relationship to the synthesis of deoxyribonucleic acid (DNA).

3) To determine whether the sequence of reactions leading to DNA synthesis differs in normal and cancer tissues.

Methods: Deoxyribosidic compounds are separated from tissue proteins, lipids and nucleic acids by making cold acid extracts of tissues, obtained under anesthesia and frozen in liquid nitrogen.

The deoxyribosidic compounds present in the acid extracts are isolated and purified by means of paper and column chromatography and paper electrophoresis. The isolated compounds are identified by means of absorption spectra, chromatographic and electrophoretic behavior, and colorimetric and microbiological reactions.

Routine assays of deoxyribosidic compounds are made with the organism L. acidophilus R-26, which specifically requires these compounds for growth.

Determinations of radioactivity are made with a commercial gas flow counter.

Deoxycytidine diphosphate choline and ethanolamine were synthesized by condensing phosphocholine or phosphoethanolamine with deoxycytidylic acid in the presence of dicyclohexylcarbodiimide.

Major Findings:

A. Further studies of microbiological assay: Recent reports by Siedler and Schweigert claimed that the growth of L. acidophilus R-26 was stimulated by uracil and not by cytidylic acid and that the growth of the organism in the presence of deoxyribonucleotides was inhibited by ribonucleotides. The latter could have important implications for work such as our own where it is necessary to detect minute amounts of deoxyribonucleotides in the presence of much larger amounts of ribonucleotides.

An investigation of these problems has shown that, under our conditions, uracil inhibits growth while cytidylic acid stimulates growth. Thus the medium originally specified by Hoff-Jorgensen, which includes cytidylic acid but not uracil is considered adequate.

A study of the effect of ribonucleotides on the growth of L. acidophilus in the presence of deoxyadenylate, deoxycytidylate or thymidylate showed that adenine-containing ribonucleotides inhibited growth, but only at high concentrations. Other ribonucleotides were without effect or stimulated growth (cytidylate).

B. Deoxycytidine diphosphocholine (d-CDP-C) and deoxycytidine diphosphoethanolamine (d-CDP-E). These compounds were isolated from the Novikoff hepatoma two years ago and during the past year C^{14}-labeled d-CDP-C and D-CDP-E have been synthesized. These compounds will be used to determine, by means of isotope dilution techniques, whether the concentrations of these compounds in hepatomas and in regenerating liver are elevated as compared to normal liver. Indirect evidence available now, indicates that this is the case.

In order to determine why the concentrations of d-CDP-C and d-CDP-E are elevated in these tissues, enzyme assay systems have been developed to study the formation and utilization of these compounds as shown by the following equations:

deoxycytidine triphosphate (d-CTP) + phosphocholine ⟶ d-CDP-C + pyrophosphate (1)

d-CTP + phosphoethanolamine ⟶ d-CDP-E + pyrophosphate (2)

d-CDP-C + diglyceride ⟶ lecithin + deoxycytidylate (3)

d-CDP-E + diglyceride ⟶ phosphatidylethanolamine + deoxycytidylate (4)

Assay conditions for reactions (1) and (3) have been established and will probably also hold for the reactions (2) and (4). A study of the synthesis of d-CDP-C by homogenates of normal and regenerating liver and of the Novikoff hepatoma has shown that the enzymatic activities of the latter two tissues, on a protein basis, is considerably higher than that of normal liver.

The enzymatic synthesis of lecithin from d-CDP-C has been studied only in normal liver. Preliminary to determining whether the lecithin synthesized in such homogenates is localised in the nucleus, as we previously postulated, the intracellular distribution of enzymatic activity was studied. This led to the surprising finding that the activity was localised in the submicroscopic particles. The activity in this fraction, however, was only 30 per cent of that of the whole homogenate and was not increased by the addition of other cellular fractions, either singly or in combination. The reason for this low recovery will require further experiments.

Significance to cancer research: Since the synthesis of DNA is an obligatory prerequisite of cell division and the ceaseless production of new cells is a cardinal feature of cancer in its malignant phase, the study of DNA formation has assumed one of the most important positions in present day cancer research.

The present studies on d-CDP-C and d-CDP-E may have a two fold importance with respect to control of cell division. Recent studies by Reichard and others have shown that the conversion of ribonucleotides to deoxyribonucleotides occurs at the diphosphate level. Since the simple diphosphates are not believed to be involved in this conversion, d-CDP-C and d-CDP-E may prove to be among the products formed. If this were true, they would play an important role in DNA synthesis, since present opinion holds that the deoxynucleotides used for DNA synthesis arise from ribonucleotides.

Furthermore, if it can be shown that the lipids formed from d-CDP-E and d-CDP-C are localised in the nucleus, these compounds would have considerable importance in the formation of the nuclear membrane and could conceivably control cell division in this fashion.

Proposed course of research: During the ensuing year, the experiments involving d-CDP-C and d-CDP-E will be continued. A comparison will be made of the ability of various tissues to synthesize these compounds and to utilize them for the synthesis of lipids. The activity of these tissues in producing CDP-C and CDP-E will also be explored.

The intracellular distribution of the enzymes involved in the synthesis of d-CDP-C and d-CDP-E and of lipids from these nucleotides (reactions 1-4) will also be studied. It is also hoped that definitive experiments can be devised to determine whether the lipids formed from deoxynucleotides and ribonucleotides have a different intracellular localisation. This goal may prove difficult to achieve because a negative result could merely indicate that appropriate test conditions had not been obtained.

Isotope dilution determinations of the concentration of d-CDP-C and d-CDP-E in various hepatic tissues will also be attempted.

Part B Included Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Rotherham, J., and Schneider, W. C.: Deoxycytidine, deoxyuridine, and 5-methyl deoxycytidine in rat urine. Biochim. Biophys. Acta 41: 344-345, 1960.

Weissburger, J. H., and Schneider, W. C.: An improved procedure for the preparation of phosphorylethanolamine and phosphorylcholine. J. Org. Chem. In press.

Honors and Awards relating to this project:

Serial No. NCI-408
1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies on lymphocytic tumors

Principal Investigator: Emma Shelton

Other Investigators: Mary E. Rice

Cooperating Units: None

Man Years (calendar year 1960):
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: To study the physiology and morphology of lymphocytic tumor cells and their relationship to the normal cells of the host. It is the purpose of these studies to sort out and examine separately some of the factors that may play a role in the invasion of normal tissues by the malignant cell.

The lymphocytic leukemias of mice provide a model system for the study of invasiveness. The cells of these tumors show marked variability in their capacity to invade host tissues located at sites distant from tumor inoculation. The survival time of the host is correlated with the extent of this invasion. The behavior of the cells is an integration of biological factors--growth rate, metabolism, locomotion rate, and immunological properties--; the integration of these factors must be considered against the background of host influence. It is the purpose of these studies

to sort out and examine some of these factors in order to assess more properly their relationship to the invasive capacity of the malignant lymphocyte.

Methods: The basic biological tool used in these studies is the ascites tumor. The free cells of the reticuloendothelial system and normal connective tissue are studied as representing the normal tissue that is in contact with the tumor cells.

Specific methods: 1) Double diffusion chamber: for details of construction and use, see 1957 annual report. The cells and fluid in the diffusion chambers were analyzed by--2)histochemical methods, involving specific staining procesures--3)chemical methods, involving paper chromatography, gel filtration, enzyme digestion, and quantitative determinations of the components and products of connective tissue--4) phase and light microscopy and cinematography--5) various quantitative and qualitative biological methods for assessing the number and type of cells being used.

Results: The major portion of the research during the past year has been directed toward establishing base lines of behavior of normal cells. Three main approaches have been taken, as follows:

1) Establishment of differences in the potentialities of similar cell populations obtained from different sites in the body: We have been able to show in previous work (Am. J. Anat. 105: 281-341, 1959) that the free cells of the peritoneal fluid of the mouse (lymphocytes, histiocytes, mast cells and granulocytes) form an organized tissue when grown in diffusion chambers. Many questions arose as to the nature of the cell transformations involved in the establishment of the organized tissue, consisting chiefly of collagen producing cells, from the free cells of the reticulo-endothelial system. We were especially interested in the lymphocyte since it represented the major proportion (75%) of the peritoneal cell population. Was the lymphocyte capable of transforming into a fibroblast? Was it possible to keep lymphocytes growing as lymphocytes without morphological transformation? In an attempt to answer these questions, we grew lymphocytes obtained from different sites in the body--namely, from the thoracic duct, from the lymph node and from the peritoneal cavity. It was found that lymphocyte populations from the thoracic duct and lymph node behaved differently from the peritoneal lymphocytes in two respects. First, the formation of organized fibroblastic sheets which regularly occurred when peritoneal lymphocytes were grown in the chambers was seen in only two of the thoracic duct lymph chambers (out of 25) and in only 8 out of the lymph node lymphocyte chambers (out of 29). Second, when peritoneal lymphocytes were grown in

the chambers, the lymphocytes rapidly became a minor element in the cell population, whereas, in the case of the thoracic duct lymphocytes and lymph node lymphocytes, the lymphocyte persisted as a major element of the population.

From this it appears that lymphocyte populations derived from the peritoneal cavity, the lymph node and the thoracic duct possess different potentialities for survival, growth or differentiation.

2) Effect of substrate upon growth and differentiation of normal peritoneal cells: In previous experiments the center element of the double diffusion chamber was a Millipore filter and all of the cell transformations described have occurred in cells growing on such a filter. We were interested in ascertaining the effect of the substrate surface upon these cell alterations. Accordingly double chambers were constructed in which glass, mica, cellophane, dialysis sac, Mylar film, and ion exchange resin in sheet form were used as the center component. The behavior of normal peritoneal cells when grown in contact with these different surfaces brought out the following pertinent facts. 1) The nature of the substrate surface materially affected the capacity of peritoneal cell populations to form an organized tissue. Cell transformations of the type described in this and previous reports were not observed on any of the above substrate surfaces. Cell transformations did occur among cells that grew in the fibrous coagulum that frequently appears in the chambers.

a) It is the physical rather than chemical nature of the substrate that is important in effecting these transformations. Cells grown on ion-exchange resin of strongly acidic (sulfonic acid groups) and strongly basic nature (quarternary ammonium groups) behaved in exactly the same manner.

b) These results strengthen the concept that cell contact and cell interaction are essential to cell transformation. The cells did not readily adhere or migrate on any of these substrate surfaces, rather they accumulated in the meshes of the fibrous gel that formed in the chambers and it was here that fibroblastic transformation was observed. The surface of the Millipore filter more closely resembles a meshwork than any of the substrate surfaces tried and this physical characteristic permits free migration and ultimate contact between cells.

3) Chemical analysis of the tissue and fluid of the diffusion chamber: The diffusion chamber method permits us to grow tissue in a heterologous host. This is not done as a trick, but as a means of obtaining more tissue and fluid for analysis by using larger chambers. Our aim is to grow mouse connective tissue reproducibly in rat diffusion chambers. We wish to establish that after 2 weeks of growth, normal mouse connective tissue cells will produce "X" micrograms of hydroxyproline and "X" micrograms of uronic acid and hexosamine, these

latter compounds being components of the mucopolysaccharide produced by the connective tissue cells. It is our purpose ultimately to test the effect of the cells of certain tumors upon this model biological system.

Much time has been spent on devising and perfecting methods and little significant data have been obtained.

We have established these facts, however:

1) Mucopolysaccharide analysis: It is essential to separate protein from the mucopolysaccharide before accurate determinations of uronic acid and hexosamine can be made. This separation is done enzymatically with pancreatin. Sephadex columns are used to free the mucopolysaccharide from small molecules such as buffer salts and amino acids. We believe that the mucopolysaccharides thus obtained will give us reproducible results--something which we have been unsuccessful in achieving so far.

Preliminary findings show that the mucopolysaccharides are not associated with the tissue itself but are found in the surrounding fluid phase. As the connective tissue grows the level of mucopolysaccharide is elevated.

Analysis of Chamber Fluid and Rat Serum

Fluid	Hexose ug./mg. N	Nitrogen	Uronic Acid ug./mg. N	Hexosamine ug./mg. N	
Empty chamber	130	531 mg.	4	151	
Non-cell side fluid	160	644	10	151)	(Two sides of a double chamber with tissue on
Cell-side Fluid	220	572	40	217)	(one side but not the other.
Serum	140	811	7	166	

2) Hydroxyproline (OHP) analysis: Quantitative paper chromatography has been used for this analysis with accurate results--as long as the protein/OHP ratio is not too high. The following results have been obtained:

Hydroxyproline Content of Mouse Connective Tissue Grown in the Rat Showing Increase of Collagen Content with Time

Number of rats.. 15	Days:	OHP: ug/chamber:
	7	217
	14	539
	28	1311

ANCILLARY PROJECTS

4) Passage of antibodies into diffusion chambers:

When lymphocytes are grown in diffusion chambers, plasma cells are formed. It occurred to us that these cells might be capable of producing antibody, but we suspected that antibody might leak out of the chamber as soon as it was formed. With the assistance of Dr. M. Fink, we investigated the passage of active and passive antibody into empty chambers placed intraperitoneally in mice. Both active and passive antibody passed into the chamber very rapidly, but the antibody titer in the chambers was always lower than that in the serum of the host mouse. Other ways of assessing the immunological potentialities of these cells must be sought.

6) Long-term study of growth of connective tissue in diffusion chambers:

Subcutaneous connective tissue from the C3H mouse has been grown in diffusion chambers for over a year. Two separate series of explants from two donor mice have been passed through 12 and 10 transplant generations, respectively, and as yet no malignant transformation has occurred. This has been checked by the technique of intraocular transplantation performed in collaboration with Dr. V. J. Evans.

Significance to Cancer Research: The lymphocytic leukemias in mice very closely resemble the lymphocytic leukemias in man. The further elucidation of the characteristics of the disease in mice should have a direct bearing upon the disease in man.

The difference in potentiality of similar cell populations obtained from different sites in the body appears significant because these are the cells that a metastasizing tumor cell would contact. It indicates that the response of these cells to the presence of the tumor might not be the same in all locations in the body. This correlates with the well-known fact that certain tumors metastasize preferentially to certain sites.

Proposed course of the research: Investigation of the relationship between normal cells and malignant lymphocytes grown in diffusion chambers will continue. Specifically, the chemistry of normal connective tissue will be used to study the effect of tumor cells upon connective tissue metabolism. We are proceeding now to isolate tumor cells in pure lines, uncontaminated with normal cells, to use for this purpose.

Study of the normal free cells of the reticulo-endothelial system will also continue. It is planned to study the effect of tumor cells upon the capacity of these cells to differentiate.

Part B Included Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Shelton, E. and Rice, M. E.: Growth of normal peritoneal cells in diffusion chambers: A study in cell modulation. Am. J. Anat. 105: 281-341, 1959.

Honors and Awards relating to this project:

Serial No. NCI-409
1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Electron Microscope Studies on Various Normal, Virus Associated, Malignant, Intact and Fractionated Cells.

Principal Investigator: R. F. Zeigel

Other Investigators:

A. J. Dalton
F. J. Rauscher
E. L. Kuff
E. Shelton
S. Wollman
M. Fink

Cooperating Units: None

Man Years (calendar year 1960):

Total:	2
Professional:	1 2/3
Other:	1/3

Project Description:

1. Type A "virus particles" of Bernhard have been observed, using the electron microscope, to bud from the apical plasma membrane of pancreatic acinar cells in the chick. We have not as yet established their viral nature by bioassay. They do, however, possess morphological characteristics which strongly suggest that they are identical to virus particles previously described in association with lymphomatosis, Rous sarcoma, erythroblastosis, myeloblastosis, myxosarcoma de Fujinami, etc.. This is the first definite evidence suggesting a "budding"

phenomena or proliferation of quantities of particles in any tissue of the domestic fowl. These characteristic morphological entities have been observed to originate in the pancreas of 5 of 12 chicks and embryos examined. Occasionally other organs were fixed simultaneously with the pancreas, including heart, liver, lung, kidney, spleen, and gut. When particles were observed to bud from the pancreatic acinar cells, all the other tissues examined contained the particles in intercellular spaces and in intra-cytoplasmic vesicles and vacuoles. In the cases where particles could not be observed in formative stages in the pancreas, neither could they be demonstrated in the other tissues examined after an extensive search. These observations suggest a possible means of transmission of particles through the G.I. tract, urinary, and upper oral and respiratory tracts. Various experimental procedures and morphological endeavors have been proposed in the light of these observations. Future investigations on "virus particle" associations with the domestic fowl are planned and will be carried out in collaboration with Dr. F. Rauscher.

2. Application of staining techniques with heavy metals in electron microscopy has proven of great value in this laboratory. Procedures developed last year resulted in a brief note in collaboration with Dr. A. J. Dalton and a demonstration of prints at the annual E.M.S.A. meeting in Milwaukee, Wisconsin. This exhibit of 8 plates was chosen to go on tour with the traveling exhibition of the E.M.S.A. Various other heavy metal compounds and modifications of techniques are being tried in our laboratory.

3. Observations on the albumen-secreting gland of the hen oviduct, using recent techniques of fixation, embedding, and staining proved successful. The secretory product is thought to be produced in close association with the ergastoplasm, accumulated in large cisternal regions and released into the secretory lumina. The Golgi regions of these cells are widely distributed, and it is suggested that this organelle is active in partially concentrating the secretory product prior to its release. Electron micrographs of this material were presented at the Gordon Conference of Cell Structure and Metabolism at Meriden, N. H. A short paper with Dr. A. J. Dalton was submitted to Prof. G. C. Hirsch for publication in his monograph in Protoplasmatologia.

4. This study involves the use of the electron microscope in analyzing various intact cells, and cell fractions prepared by Dr. E. L. Kuff. Observations on ribonucleoprotein particles of the Novikoff hepatoma and on the Golgi material of a plasma cell neoplasm have been made. Electron micrographs of these materials have been published and presented by Dr. Kuff at the Gordon Research Conference on Cell Structure and Metabolism. Further collaboration is planned concerning the ultrastructure of other fractions from various cell types. This investigator feels that this association is well founded in that observations on both structure and function are closely integrated.

5. In collaboration with Dr. S. Wollman a variety of transplantable rat thyroid tumors are now being investigated with the electron microscope. A considerable amount of information has been accumulated in Dr. Wollman's laboratory concerning their biochemistry which warrants correlation with their micromorphology. This project has only recently been initiated.

6. Further investigations on cells grown in tissue culture (Millipore filter) chambers under the direction of Dr. E. Shelton are in progress in this laboratory. Various aspects of the ultrastructural characteristics of cyto-differentiation and collogen formation may be elucidated.

7. In collaboration with Dr. F. Rauscher and Dr. M. Fink, we plan to study various immunological and serological aspects of certain avian viruses as regards their ultrastructural associations. Specific staining and localization of various components may be involved.

8. This part of the project involves the revision of a manuscript concerning the embryogenesis of the chick pancreas. The fact that this tissue may be important in the story of avian virology warrants its completion.

9. A portion of laboratory time was expended in assisting a visiting scientist in maintenance and operational techniques of the electron microscope.

Part B included Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Zeigel, R. F.: Morphological evidence for the association of virus particles with the pancreatic acinar cells of the chick. Submitted to J. Nat. Cancer Inst.

Dalton, A. J., and Zeigel, R. F.: A simplified method of staining thin sections of biological material with lead hydroxide for electron microscopy. J. Biophys. Biochem. Cytol. 7: 409-410, 1960.

Kuff, E. L., and Zeigel, R. F.: Cytoplasmic ribonucleoprotein components of the Novikoff hepatoma. J. Biophys. Biochem. Cytol. 7: 465-477, 1960.

Zeigel, R. F., and Dalton, A. J.: Extended observations on the ultrastructure of the albumen secreting cells in the hen oviduct. Submitted to Protoplasmatollgia, Prof. G. C. Hirsch.

Honors and Awards relating to this project: None.

Serial No. NCI-414
1. Laboratory of Biology
2. Cellular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Cancer research with the use of diffusion and transparent chamber methods

Principal investigator: Ruth Merwin

Other Investigators: Thelma Dunn

Cooperating units: None

Man Years (calendar year 1960):
Total: 4
Professional: 2
Other: 2

Project Description:

Problem I.--Induction of plasma-cell tumors and sarcomas by diffusion chambers placed intraperitoneally in mice. R. Merwin and T. Dunn.

Objectives.--To determine what the factors are that are involved in the induction of these tumors.

Methods.--Diffusion chambers or parts of them or materials of which they are made are placed intraperitoneally and the mice observed for the development of tumors. Many of the tumors are transplanted until a diagnosis is obtained and some are transplanted to be sure that the tumors arise from the hosts and not from the tissues in the chambers.

Major Findings:

1. A difference between the BALB/c and C3H strains of mice was found. More of both types of tumors were found in the BALB/c's. The two strains also differed in the type of host reaction to the presence of the chamber.

2. Both plasma-cell tumors and sarcomas show a spotty distribution; that is, more are found in some experiments than others.

The important factor has, as yet, not been found although a number of leads suggest further experiments.

3. The appearance of sarcomas shows no marked correlation with the size of the pores in the millipore membranes, with the use of pen-strep, with the use of stilbestrol nor with the presence or absence of tissue within the chambers.

4. A number of factors present or probably present in the original experiments in which plasma-cell tumors were induced have been shown to be non-essential for the tumor induction. These non-essential factors are: the presence of tissue inside the chamber, stilbestrol, infection. Further, no one of the chamber parts is essential, neither the membrane, the lucite nor the adhesive.

5. Mice inoculated as newborns with a preparation that would be expected to contain virus if the usual type of virus were present have not, at 1-1/2 years, shown any abnormal tumor development.

Proposed course:

1. To continue experiments to determine what the crucial factors are in the induction of plasma-cell tumors and sarcomas.

2. To study factors controlling the transplantability of the plasma-cell tumors. Some tumors may arise from inoculations a year after the first have appeared.

Significance to cancer research:

Plasma-cell tumors have until recently been relatively rare in laboratory animals but they are not rare in humans and any increase in an understanding of the cause and character of these tumors is of value. The tumors are being checked by Dr. Fahey to find as many types of tumors, with regard to abnormal protein production, as possible for comparison with the variety of human plasma-cell tumors.

Problem II.--Survival of cells inside diffusion chambers.

Objectives.--To find out whether tumor lines can be maintained without change by placing them in diffusion chambers instead of constantly transferring them.

Methods.--After tumor tissue had been in diffusion chambers for 6 months to 2 years the membranes were punctured and left in place or the chamber was opened and placed in a new host.

Major Findings:

It seems probable that the method is usable. Sarcomas, Lymphoma-2,

L1210, mammary tumors and plasma cell tumors have been tested. No evidence of adaptation has been found except for tissue culture cells.

Significance to cancer research:

This method would be an easy one for maintaining a number of tumor lines.

Proposed course:

Possible changes in histological characteristics and in the production of abnormal proteins is being studied.

Problem III.--Virus studies using diffusion chamber techniques.

Objectives.--To place tumors of known viral etiology in diffusion chambers and to determine what size pores the virus will pass through and how infective such an introduction of the virus will be. Also to test tumors not known to be associated with a virus to see if a virus might be detected.

Methods.--Diffusion chambers with the tumor in question inside were placed in mice which were observed for the development of tumors in greater numbers or of a different type that those developing in mice with other tumors within the chambers.

Major Findings:

Mammary tumor agent.--The agent passes through membranes with pores .45 micra in diameter and not through pores .1 micra or smaller. Tumors appeared in hosts of the larger pored membranes whether the host was a BALB/c- (without the agent) and the donor a C3H+ or the host was a C3H- and the donor a BALB/c+. No tumors appeared in BALB/c- mice with C3H- mammary tumor tissue in the chambers.

Polyoma.--No tumors other than sarcomas appeared in the SWR mice carrying this tumor in diffusion chambers. The role of the virus in inducing the sarcomas is not certain as not enough control SWR mice have been studied.

Moloney leukemia.--The virus passes through membranes with pores .45 micra in diameter but does not seem to pass through the smaller pored membranes.

Other tumors.--No evidence of tumor induction was found for the plasma cell tumors induced in the diffusion chamber experiments; for several sarcomas, including S-37; nor for Lymphoma 2. However, the undifferentiated plasma-cell tumor 70429 may have some capacity to induce tumors.

Proposed course:

Moloney leukemia.--If it is found that there is anything atypical about the infection obtained with the diffusion chambers more will be done with this virus.

70429.--Will repeat experiments on 70429 to see if further evidence of the induction of tumors in the mice containing this tumor in diffusion chambers can be found.

L1210.--Will investigate the finding reported by Dr. Algire indicating that L1210 cells pass through membranes with pores .45 micra in diameter as this does not seem likely.

Problem IV.--The effect of local irradiation on tumors studied in vivo in transparent chambers.

Objectives.--To study the interaction of tumor and host tissues during regression and regrowth of relatively radio-resistant tumors after irradiation.

Methods.--Grafts were observed in vivo in transparent chambers and photographs were taken.

Major Findings:

Five recently induced sarcomas tested in an effort to find radio-resistant tumors were found to be radio-responsive. Evidence was found that at 3,000 r of x-irradiation an occasional area of endothelium does not permanently lose its capacity to grow.

Significance to cancer research:

X-irradiation is an important means of controlling tumor growth, yet much of the basis for tumor treatment is still empirical.

Proposed course:

To test a new, relatively slow-growing tumor for its degree of radio-resistance. Also to allow the more radio-resistant of the tumors tested to grow larger so they will be more resistant. Hair growth which makes visibility poor and photography difficult has interfered with getting tumors of the desired size, however methods of controlling the hair growth are being tried.

Part B included Yes No X

Serial No. NCI-417
1. Laboratory of Biology
2. General Biology Section
3. Bethesda 14, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Investigation of the role of genes and their relationship to non-genetic factors in the development of cancer.

Principal Investigator: W. E. Heston

Other Investigators: Margaret K. Deringer and George Vlahakis, with collaborative research with Arnold W. Pratt, A. J. Dalton, and J. B. Moloney.

Cooperating Units: NCI-421

Man Years (calendar year 1960):

Total:	6
Professional:	2
Other:	4

Project Description:

Objectives: To ascertain the role of genes in the development of tumors in mice, and to relate the genetic effects with those of other biological, chemical, or physical agents in the causation of cancer in mice. To identify and locate specific genes influencing the occurrence of various kinds of tumors and to determine the gene-action pathways through which their effects become manifest.

Methods: In contrast with much of the former work on the genetics of cancer, much of our work now is directed toward the analysis of effects of specific genes on the various types of cancer rather than working merely with strain differences where specific genes have not been identified. Genetics of the mouse has developed to a point where this approach with specific genes can profitably be pursued. There are now 87 or more gene loci of the mouse identified in 19 linkage groups. These may represent all but one of the chromosomes. Nine of these loci have been shown to be linked with neoplasia.

At the present time two of these loci are of particular interest to us: the obese gene ob in linkage group XI that results in excessive obesity, and lethal yellow A^y in linkage group V that is lethal when homozygous, but when heterozygous causes the coat to be yellow and causes an increase in body size. We have the Vob line carrying the obese gene ob, and lethal yellow A^y is in our strain YBR. The great advantage of A^y is that because of forced heterozygosis at this locus during the long period of inbreeding, when yellow YBR mice are outcrossed to any other inbred strain the F_1 hybrids produced are isogenic in that they are genetically alike except that half of them carry the lethal yellow gene and are yellow, and the other half carry the non-agouti allele and are non-yellow. Thus, in these F_1's we can analyze the effect of this specific gene, for any difference in occurrence of tumors in the two color types is known to be due to this gene.

A further advantage in studying the effect of the lethal yellow gene in these F_1 hybrids is that tissues can be transplanted freely between the two color groups. We have tested the A^y gene with skin transplants and it has no histocompatibility effect. This enables us after identifying an effect of the gene on any type of tumor to try further to identify its path of action through transplantation of tissues or organs. For example, after showing that the gene affected occurrence of mammary tumors, the next step was to ascertain by reciprocal transplantation of ovaries between the two color types whether or not the effect of the gene was manifested through the ovary. This was first done with young adults, but we have also worked out techniques for such transplantations in day old females with low temperature anesthesia. The transplantation can be followed by breeding tests in these F_1's to ascertain by observing the color of the offspring whether or not the transplanted ovaries are functioning. Pituitary glands are also transplanted in these hybrids and in other strains, placing the transplanted gland in the subcapsular area of the kidney. In some cases hypophysectomy is done without receipt of a transplanted gland.

Since homozygous obese ob ob mice are sterile, the influence of this gene on occurrence of tumors has been studied in the F_2 generation produced by outcrossing heterozygous animals of the Vob line and mating the resulting F_1 hybrids that have in turn been shown to be heterozygous by breeding tests. During the past year F_2's resulting from outcrossing Vob mice to strain C3H have been studied for linkage between ob and mammary tumors in the females and hepatomas in males, and F_2 s resulting from outcrossing Vob mice to strain A have been studied for linkage between ob and spontaneous pulmonary tumors.

We have continued to attempt to correlate the occurrence of neoplasms with normal growth and to study the effect of these specific genes on each. Measurements of normal growth have included body weight, body length and length of femur both of which are expressions of skeletal growth, and weight of the dissected out gastrocnemius muscle an expression of muscular development.

Standard statistical procedures are used in analyzing the data. Significances of incidence differences are determined by the chi square test and differences in mean number of tumors by the t test. Pearson product-moment coefficients of correlation have been determined. This is one of the standard procedures for determining correlation and is applicable to our data.

Results:

The data on the effect of the A^y gene on mammary gland tumors and hepatomas in (C3H X Y)F_1 mice have been written up and presented for publication. The A^y gene increased susceptibility to mammary tumors in that it caused the tumors to occur in the virgin females with the gene when they were only about half as old as the virgin females without this gene were when they got their tumors. The final incidence in both groups was approximately 100 percent. Breeding the females eliminated this difference. The tumors came up in both the breeding females with the gene and those without it at the same age at which they came up in the virgin females with the gene and in both groups the incidence approximated 100 percent. Thus, the A^y gene seems to have the same effect on occurrence of mammary tumors as breeding has, which suggests that the effect of the gene may be manifested through the ovary.

Reciprocal transplantation of ovaries between F_1 females with the gene and those without it have failed to implicate the ovaries but the data are incomplete and all observations thus far have been on females that had their ovaries exchanged as young adults. We are now transplanting ovaries in day old F_1's to see if by making the exchange at this early age we can link the effect of the gene to the ovary. If not, we should then go to transplantation of the pituitary glands or possibly to transplantation of the hyperplastic nodules of the mammary gland to see if the action of the gene is linked with the pituitary gland in stimulating the mammary gland or with the mammary gland in controlling its neoplastic response.

Among the mammary tumors of these (C3H X Y)F_1 females we have observed a histologic type rarely observed in other strains or hybrids. It is composed of epithelial cells surrounding angular spaces and has an abundance of stroma that takes the Van Geisen stain for collagen. Since these tumors have not been observed in our high tumor strains, but do occur in these F_1 hybrids resulting from outcrossing to strain YBR we have labeled this class of tumors as adenocarcinoma, type Y. All but one of these new tumors occurred in breeding females suggesting some hormonal factor in their etiology. They occurred at an average age comparable with that of other types of mammary tumors and they were not influenced by the A^y gene.

The effect of the A^y gene on hepatomas was noted in the (C3H X Y)F_1 males. Males with the gene had a significantly higher incidence and average number of spontaneous hepatomas than those without the gene.

However, the incidence was high in both groups. The feeding of carbon tetrachloride had no detectable carcinogenic effect on inducing hepatomas in these highly susceptible males and there was no evidence of any effect of the A^y gene on hepatomas in these males fed CCl_4.

In these (C3H X Y)F_1 hybrids the A^y gene increased body weight by about 10 grams in the males and 15 grams in the females. It significantly increased body length in both males and females and muscle weight in females. Thus, this gene that increases normal growth increases susceptibility to mammary tumors, hepatomas, and as shown previously pulmonary tumors. Significant positive correlations were found in the males between number of hepatomas and body weight, body length and length of femur.

In an attempt to throw more light on the manner in which this A^y gene that increases body size is increasing occurrence of spontaneous hepatomas, we have hypophysectomized approximately 100 of these (C3H X Y)F_1 males with and without the A^y gene, and we will observe the occurrence of hepatomas in them in comparison to occurrence of hepatomas in nonoperated controls. Since so nearly 100 percent of these males get hepatomas when fed our NCI pellets, we have fed these hypophysectomized males and their controls on Purina chow that reduced the incidence of hepatomas in C3H males.

The study of the effect of the obese gene ob on occurrence of spontaneous pulmonary tumors has now been completed. Corresponding with the published results of the effect of the gene on induced pulmonary tumors, ob significantly decreased the occurrence of spontaneous pulmonary tumors. Since the obese ob ob mice weighed much more than their normal littermates it would seem at first that these data were contradictory to the other data, but the heavy weight of the obese mice is due to the large amount of adipose tissue rather than skeletal and muscular tissue. When growth of muscle and skeleton was considered it was found that the mice with tumors were larger than those without tumors.

Results of the study of the effect of the obese gene ob on occurrence of hepatomas are irregular. This study was carried out on (C3H X Vob)F_2 males. More of the obese mice had hepatomas than of their normal sibs, and the obese mice had a higher average number of hepatomas. This result is opposite to the effect of this gene on pulmonary tumors. Furthermore, there does not appear to be a positive correlation between number of hepatomas and body measurements other than weight. The only significant differences observed were a higher muscle weight in the normal sibs than in the obese mice and a greater femur length in the normal sibs.

In the (C3H X Vob)F_2 males that received CCl_4 there was not a significant difference in occurrence of hepatomas between the obese ob ob mice and the normal sibs. Thus, the CCl_4 blotted out the effect of the obese gene on hepatomas just as it did the effect of the A^y gene on hepatomas.

The study of the effect of the obese gene on mammary tumors in (C3H X Vob)F_2 females is not completed since a few of the normal females are still living. There are enough data, however, to indicate what the outcome of the experiment will be. The normal females will have an incidence of mammary tumors of 60 percent or more whereas that of the obese females will be approximately 24 percent, but the average tumor age of these obese females is much less than that in the normal females. Since obese females do not breed it is not surprising that they have a much lower mammary tumor incidence. It is surprising, however, that the tumors that do occur in them appear at an early age.

In the study of the F_2 hybrids between strain A and strain LG that was selected for large body size, all the mice have been autopsied. The purpose of this study was to see if there would be any correlation between occurrence of induced pulmonary tumors and measurements of body size in these hybrids that we expected to show great variation due to segregation of many unidentified genes for size. There was a great variation in induced pulmonary tumors as would have been expected in these F_2 hybrids, but there was surprisingly little variation in body size. All of them were large. Furthermore, the group failed to show any correlation between number of pulmonary tumors and body size. The data for these F_2's, the F_1's, and the parent strains will be analyzed further from the standpoint of inheritance of body size, for they may yield some new information on this point. It is hard to understand how when we outcrossed strain A to a strain with large body size presumably because of selection of genes for large size, we did not get variation in the F_2 ranging at least to the means of each parent strain.

The isolation and demonstration of a leukemia virus by Moloney from sarcoma 37 interested us in ascertaining whether or not such an agent might be harbored in some of the tumors that we have been transplanting in some of our strains for a number of years. In collaboration with Dr. Moloney, therefore, cell free extracts of our 8 transplantable tumors were prepared and injected into newborn mice. These tumors included 4 pulmonary tumors, one squamous cell carcinoma that originated in the forestomach, one sarcoma of subcutaneous origin, and one tumor that was originally a bone tumor. Although the results to date are negative, the injected animals are still alive and are continually being observed for tumors. In preparation of all of these extracts the tumor material had been subjected to centrifugation and filtration. We have now recently altered our procedure by using differential centrifugation alone thereby recovering more material, and possibly more agent.

During the past year we have incorporated into our breeding program for our strain C3H and strain C3Hf mice a systematic program for testing four mouse diets. These include our NCI pellets that we have been feeding for a number of years and that are made according to Dr. Harold Morris' formula, the Old Guilford diet, a commercial diet similar to the NCI pellets, and an NIH simplified diet and an NIH modified diet, the formulas of which have recently been drawn up by Dr. Morris in an attempt to get a

satisfactory diet that will be less expensive than the other two. We are testing these diets in respect to breeding capacity of the mice over a number of generations, and growth of the mice. The tests are completed through the 4th litter of the first generation of the C3H and the 3rd litter of the first generation of the C3Hf. We have some information on the second generation. Thus far it appears that all diets are good, but that the Old Guilford diet may be the best. Its apparent superiority over the NCI pellets may merely be that its pellets are softer. When these tests have been completed we will be able to choose the best diet from reliable data.

Significance to the Program of the Institute:

A cancer is a clone of living cells growing without normal morphogenic controls in a living host. It is not a cell growing in a tissue culture flask, a chemical in a test tube, a bacterial test for enzymatic activity, or an electron microscope picture of a virus. Any research program of an institute for cancer research must, therefore, include some investigators who are primarily interested in the living animal, in the intrinsic and extrinsic factors that cause tumors to arise in that living animal, and in the nature of the neoplasm that arises. In our program we are interested in the living animal from the time it is conceived until it has died from the neoplasm. We are interested in the genotype that is directing the developmental processes and in the relationship of these genes to hormonal factors, nutritional factors, chemical and physical carcinogens, and viruses that are involved in the etiology of the cancer. We are interested in the relationship of the etiology of the cancer to its histologic type and pathogenesis. Such programs form the core of cancer research and serve to tie together information gained through fringe approaches.

We now have a rather impressive amount of data on the role of specific genes in the etiology of cancer. In fact we have made much more progress in this more definitive area than has any other laboratory. We will be able to continue to make such progress not only in determining what tumors specific genes affect, but also in revealing something of their paths of action--the organ or tissue sites through which their effects become manifested. This will be of importance in an attack on the problem on the molecular level. Strain differences could be studied on the molecular level without having identified the genes involved, but, of course, a much more definitive study could be made when one is studying a single gene difference; when he knows the gene involved; and when he has some idea of the organ or tissue in which it is acting. The lethal yellow gene will be of particular importance not only because it affects the occurrence of a number of different tumors, but also because the F_1's resulting from outcrossing the strain YBR to other strains provide an ideal system for such a study in that they are genetically alike except for this one gene. Until lines isogenic for other known genes influencing the occurrence of tumors have been developed, there will be no other such ideal system.

Proposed course of the project:

We will continue to study the living animal and the intrinsic and extrinsic factors that determine whether or not a tumor will occur in the animal and the nature of the tumor that arises. The work will continue to be focused on the factors present from conception, the genes, and in relating these factors to other factors in the etiology of the neoplasm.

Various facets of the program now underway that have not been completed will be carried to completion. The study of the effect of the A^y gene on pulmonary tumors when the growth of mice with the gene is controlled through dietary restriction will be completed. A correlative of this study is the one in which the A^ya mice and their a a sibs have been injected with gold thioglucose to increase body weight, and the observation of the effect of this treatment on pulmonary tumors. We will continue to study the path of action of this gene on influencing the occurrence of mammary tumors through studies of transplantation of ovaries, hypophyses, and possibly hyperplastic nodules of the mammary gland.

Since the A^y gene causes mammary tumors to occur in virgin females with the mammary tumor virus much as they occur in breeding females, we are producing a group of (C3Hf X Y)F_1 females without the mammary tumor virus to see if in the absence of the virus this gene also has an effect on occurrence of mammary tumors comparable with the effect of breeding. Here the effect should be detectable on incidence as well as on tumor age.

To attempt to gain some information on the path of action through which the A^y gene affects the occurrence of hepatomas we have hypophysectomized (C3H X Y)F_1 males and these will be studied for occurrence of hepatomas and compared with intact (C3H X Y)F_1 males.

To study the effect of extra pituitary glands on mammary tumor development we have transplanted extra pituitary glands beneath the kidney capsule of C57BL mice, some receiving 5 extra glands and some only one. Both males and females have been subjected to the treatment after which the males have received a subcutaneous pellet of stilbestrol. This study will extend throughout the coming year or longer.

In collaboration with Dr. A. J. Dalton we have started an electron microscopic study of the mammary tumor agent. We are supplying and will supply Dr. Dalton with mammary tumors from the following kinds of mice: C3H with the agent, C3Hf or C3He without the agent, DBA females with the agent, DBAe females without the agent, C57BL females with the agent, C57BL females without the agent but with tumors induced with added pituitary glands, A females with the agent, and Af females without the agent. In the last group we may need to use a carcinogen to induce the tumors. We hope to be able to choose females in each case who have nursed susceptible young so we will have a biological test for the presence or absence of the agent. It will take several years to get all of these types

of females with suitable tumors, but with the very excellent pictures Dr. Dalton gets (he is about the best electron microscopist in the world) we hope eventually to be able to clarify the question as to just what is the mammary tumor agent as seen in the electron microscope.

At the request of Dr. Simon Iverson at the Royal Beatson Memorial Hospital in Glasgow, we are working up some of our extensive data on occurrence of mammary tumors in C3Hf females without the mammary tumor agent. Dr. Iverson hopes then to correlate these data with similar data that Dr. Pullinger in Glasgow has, and we hope that from this some light may be shed on mammary tumor carcinogenesis.

In the study on the effect of concentration of oxygen on induction of pulmonary tumors in collaboration with Dr. Arnold Pratt we hope during the year to conclude the description of dose response curves. When Dr. A. C. Allison of London returns to the Institute in October, he wants to collaborate with Dr. Pratt and us in studying the effect of carbon monoxide on induction of pulmonary tumors much as we have studied the effect of oxygen.

Part B included Yes X No

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Heston, W. E. and Vlahakis, G.: Influence of the A^y gene on mammary gland tumors, hepatomas, and normal growth in mice. J. Nat. Cancer Inst. (In Press.)

Heston, W. E.: Neoplasia, chapter. In Methodology in Medical Genetics III, Methodology in Mammalian Genetics, W. J. Burdette, ed., Columbia University Press. (In Press.)

Heston, W. E.: The genetic concept of the etiology of cancer. Proc. Fourth Nat. Cancer Conference. J. B. Lippincott, Philadelphia. (In Press.)

Serial No. NCI-418
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Host-tumor relationships

Principal Investigator: Morris K. Barrett

Other Investigators: Edward J. Breyere, Malcolm B. Melroy
and, in collaboration, Margaret K. Deringer

Cooperating Units: NCI-421; NCI-427(c); NCI-410; NIAID-25
(Dr. Du Buy)

Man Years (Calendar year 1960):
Total: 5
Professional: 3 (Dr. Breyere is on a fellowship)
Other: 2

Project Description:

Objective: Our working hypothesis concerns a field presently called "immunogenetics" which covers an area of overlap between the two classical disciplines but seems to differ significantly from each. This is a relatively new field and many borderlines and concepts are hazy, but it appears to touch upon several obscure areas in biology, e.g., adaptive enzyme formation, embryonic induction, differentiation, irradiation protection, and the broad but ill defined field of "tumor immunity."

It is becoming increasingly apparent that biological interactions involve some undiscovered "information" or "recognition" system of general application. Our hypothesis assumes that some such system is involved in the intercellular reactions of normal differentiation, growth, and physiology and that the abnormal differentiation and growth in cancer represent an aberration in such a system. We also entertain the notion that such a breakdown may not always be complete and that therein may lie the explanation of spontaneous regression of cancer, temporary remissions of cancer, and the apparent control of metastasis and recurrence.

More specifically, we are attempting to discover what "tissue immunity" and "transplantation antigens" are. At first thought they may appear to be rather nonsensical laboratory playthings with no real place in natural phenomena. However, they are so widespread in nature that, speaking teleologically, they must have some physiological function. In fact, we have found some evidence that they may play a part in the physiology of gastric digestion and of gestation. We believe that discovery of the true nature of the "antigen" and the "immunity" in this field will open a new approach to many problems in cancer, both as to the intrinsic nature of neoplasia and as to its control. The importance of immunogenetic phenomena in chemotherapy is not sufficiently appreciated.

Methods: The basic procedure is transplantation of standardized tumors, or skin, into hosts of known, but highly varied genetic constitution and observing the effects of experimental variation of hosts, or tumors, or both and of immunizing injections, treatment of the antigens, effects of chemical or physical agents, etc. The disciplinary techniques are those of genetics, immunology, and experimental surgery.

Major Findings: In relationship to what is variously called "adaptation" of a tumor, or the "F_1 hybrid phenomenon," or the "Barrett-Deringer phenomenon," none of which is a good term, there have been no major positive findings to change viewpoints previously expressed. Another tumor "adapted" but only poorly and was dropped because of technical difficulty.

Another "adapted" tumor, which probably got that way by "immuno-selection" and has a different genetic relationship from the one previously reported has been obtained. It reacts to the immunity induced by blood, as expected. Thus, whatever happens when a tumor adapts it does not involve a loss of the usual response to tumor immunity. Another tumor has undergone the "hybrid phenomenon" but turned out to be a difficult tumor to work with. An "adapted" subline of a tumor that had been maintained in back-cross hosts for nearly 200 generations was tested for its responsiveness to tumor immunity. It responded to immunity induced by normal organs of the strain of origin; thus it had not become immunologically indifferent. With respect to a hybrid fetus we have observed no evidence of a reaction to the pure strain mother - but the mother is changed, as will be mentioned later.

Over a period of years there have been several indications that the Histocompatibility-2 (H-2) complex of antigens might have some special features with respect to these phenomena. Our outcrosses involved differences at this strong locus but the results generally suggested that whatever transplantability changes occurred did not involve changes in this antigen complex. In some ways this seems odd. We are in the early stages of work with new tumors. In this case the tumors and animals are all alike at this locus. The thought is that if changes occur they must be based on other loci. One of the tumors is now growing in about 50% of the foreign strain but the results suggest that what we are getting is "immunoselection" and not "adaptation." If this is so, we will have an interesting opportunity to try the effect of other outcrosses on the same tumors. This might throw some light upon the relation of "the hybrid phenomenon" to "somatic mutation," "transduction," and the like. Re-examination of some old data has convinced us that even when an adapted tumor has come to a "one factor" state and has not changed with respect to H-2 its behavior is such that multiple factors must be assumed. Also, our results with tolerance in the parous female do not depend on this locus (of which more later).

We have had a long standing interest in the "individuality" of tumors. The varied behavior mentioned above is one aspect of this. Another is our tentative observation that in some combinations tumors may not, as is generally believed, grow as well in the F_1 hybrid as in the parent strain. We think that we have seen this 3 times. If it is true it may require some revision of the simple concept of "one gene--one antigen" with "complete dominance." Along with this we have made careful, frequent measurements of a number of spontaneous mammary tumors in mice. The expected extreme variation in growth was observed among mice but those variations were no greater than those between multiple tumors on a

single mouse. The latter can hardly be caused by the hormonal or metabolic status of the host. However, we have obtained 2 very slow growing uterine tumors of mice. We have tried hormonal stimulation of one of these and found that it is enhanced by stilbestrol but not by progesterone, testosterone, or cholesterol.

With respect to the question of whether "adaptation" involves the introduction into the cell of something like DNA protein, a virus, a plasmagene, or the like; we have been cooperating with Dr. Du Buy on a small project. He has a technique for adsorbing such material on very fine ground glass particles and then propelling it through a cell mass in the centrifuge. The idea is to extract possible transducers from one strain, inject them into tumor cells of another strain, and see if the cells are changed in their interaction with the first strain. So far there has been much technical difficulty and few results but those obtained encourage persistence.

A naturally occurring, physiological aspect of immunogenetics has been observed in a study of multiparous female mice. In panmictic and therefore genetically heterogeneous populations (such as in man), tissues exchanged between its members are almost always rejected as the result of an immune reaction directed against the graft. Two notable exceptions are fetuses and tumors of chorionic origin in women. It seemed possible to us that some alteration in the immune mechanism of the mother may have occurred to allow the survival of either of these antigenically foreign tissues. In the course of our experiments in mice, we have found that a state of immunological tolerance is induced in the mother as a consequence of specific parity. To date, this effect has been observed in 3 genetic systems with 3 transplanted tumors and with skin homografts in 2 combinations, and it can be seen whether the materials are the same or different at the H-2 locus.

This tolerance is highly specific and depends upon a genetic concordance between the transplanted tissue and the sire of previous litters, i.e., it was observed in postpartum mice parous by males of the strain from which the grafts originated. Tolerance was not found in females parous from intrastrain matings, nor in virgins, nor in those parous by males of still another strain unrelated to the graft. Experiments designed to explore the nature and mechanism of this phenomenon have thus far shown the following. (1) Tolerance increases with multiparity. (2) It is effective both in the case of "natural" and induced resistance. (3) It is long lasting, i.e., at least 150-200 days in the mouse. (4) A previously induced immune state can be partially abrogated by specific parity. (5) This

tolerant state is resistant to the usual immunization procedures, i.e., immunity cannot be induced in tolerant females by an inoculation of homologous blood and a degree of tolerance remains even after the rejection of a homologous skin graft. (6) The effect of parity is not due to mere exposure to the male in caging, and (7) the presence or absence of the mammary tumor milk agent in the male had no detectable influence.

In one of the tumor-host combinations studied, a unique migration of a transplanted tumor was observed in tolerant females. Initially the tumor grew at the site of inoculation until it had reached a moderate size. It then became umbilicated in the center (not necrotic). This process continued until no tumor was grossly or microscopically visible at the inoculation site. Thus the tumor grew in the form of a doughnut, and increased in size by growth at the periphery accompanied by an apparent simultaneous regression in the center. This observation might suggest a local immunity, but this has not as yet been determined. The tumor does not grow this way in the strain of origin.

This problem seems at present to be getting more complicated. There are several indications that some of our ideas may be too simple and direct. It appears to us that skin grafts may permit an approach to problems for which tumor grafts are unsuitable, but we are not satisfied with present techniques for skin grafting. Accordingly we are trying to develop technique for obtaining dependable results when many grafts are placed simulatneously on a single recipient. Much progress has been made and we believe that the objective can be reached. If this can be done it seems possible to set up experiments, involving "crisscross" and "round robin" types of transfer, that will permit observation and mathematical analysis of multifactor effects in a way not heretofore possible. Thus far, fair success has been attained with as many as 20 grafts on a single mouse. As an incidental finding, we have confirmed the reported relative incompatibility of a male graft on a female recipient within strain C57BL.

Collaborative preliminary studies with Dr. Moloney have indicated that tolerance induced by parity may decrease the latent period of the leukemia virus extracted from Sa 37. These studies are being continued and the results to date are only suggestive.

Some strains of mice appear to be more resistant to the development of virus induced tumors than other strains. Therefore, if a virus induced tumor arose naturally in a relatively resistant strain, the detection of the virus may be difficult.

In the case of one tumor, 3 previous attempts (by Dr. Moloney) to extract an active virus gave negative results. We have since taken females of another strain, which we believe to be generally more susceptible to tumor viruses, and grown this homologous tumor in them through the action of the tolerance induced by parity. After growth in this new strain (which we hoped would favor virus replication), an extract was prepared by Dr. Moloney and newborn mice were inoculated. To date, only about 1% of the animals have shown encouraging results. The induced tumor appears to be similar to the original tumor in its growth patterns and morphology.

One of our tumors has a notable ability to induce a conspicuous blood supply at and around the site of implantation. We wondered whether this was a special property of the tumor. Accordingly tumor and embryo pulp were implanted simultaneously at separate sites. The tumor and embryo were of the same strain but the hosts were same or different strain. Technical difficulties were encountered and the results were inconclusive but an interesting incidental observation was made. The embryoma differentiates, stops growing, and regresses in both hosts but differentiation was noticeably more advanced when the strains were the same.

After our first presentation of "maternal tolerance" we were told that the records of the breeding colony at Bar Harbor revealed an increase in male to female ratios in late litters as compared to first litters. This could be viewed as another natural manifestation of maternal tolerance (male grafts are incompatible with female recipients in some, if not all, strains). Accordingly we examined Dr. Deringer's records. Such an effect was found in earlier records but it did not persist in later records. We decided to leave this type of investigation to others.

Acting on an original suggestion of Gamow and with the advice and encouragement of Dr. Stetten, Mr. Eck has spent considerable time in exploring the feasibility of approaching the problem of protein structure by a cryptogrammatic analysis of sequences. Preliminary tabulations and calculations give some encouragement but are inconclusive.

In discussions of the relationships of "transplantation immunity" to bacterial immunity and to tuberculin sensitivity one criterion often mentioned is that transplantation immunity, once established, cannot be abrogated by irradiation. We are not so sure of this and consider it sufficiently important for moderate reinvestigation. In collaboration with Miss Uphoff we are beginning experiments using preimmunized mice, lethally irradiated, and protected with bone marrow. Early results are

inconclusive owing to technical difficulties, but there is some suggestion that the generally accepted result is not correct.

Mr. Melroy has spent the year examining mice from all parts of the Laboratory of Biology for the presence of polyoma virus by means of the hemagglutination-inhibition technique. He is making a separate report.

A moderate amount of time has been spent refereeing manuscripts for the J. Nat. Cancer Inst., Cancer Research, and occasionally for others.

I participated, by invitation, in symposia at the N. Y. Academy of Sciences and at Jackson Memorial Laboratory, and delivered a Ruben Kahn Memorial Lecture at Ann Arbor.

Significance: The field with which this work is concerned is so difficult and obscure as to make clear definition and outlines almost impossible. Nevertheless, there is a slowly growing body of evidence which indicates that there are interactions between tissues and their host which involve critical factors in the phenomena of cancer, embryology and transplantation of normal tissues. A defensible definition of cancer cannot be given today except in descriptive biologic terms. The terms usually included things related to the experiments that we have been describing. The experiments may be significant in several ways both practical and theoretical.

The most important implication is that some of these results suggest progress toward understanding of what controls the growth of a cancer and how this differs from normal tissues. We have already seen that a tumor's power to overcome host resistance can be altered; its rate of growth, tendency to regress, and tendency to metastasize are all subject to experimental change. The host's reaction can be altered experimentally, not only between similar hosts, but also at different times and sites in the same host. The changes in the tumor have eluded morphologic, chemical, or serologic definition. One of the antigens which changes the host would be missed by current chemical methods. An important aspect of the "hybrid phenomenon" is its possible relations to "transduction." Important possibilities with regard to the effects of parity can be imagined in relation to several complications of pregnancy including Rh disease and choriocarcinoma. Interpretations are unsure. However, one might attain a biologic description of the factors that sometimes cause spontaneous tumors of man and animals to regress and that control metastasis. It has already been observed in this

laboratory (and confirmed elsewhere) that such factors materially affect the outcome of many chemotherapeutic experiments.

Relation to others: There is a complex theoretical interlocking between this work and the work of Drs. Smith, Law, Merwin and Sanford, and Miss Uphoff. Many of the results attendant upon the use of Millipore filters were foreshadowed by the work on the nature of the antigen in tumor immunity. The work on adaptation has a strong relation to recent developments in radiation biology and in tissue transplantation.

Proposed course: Extension on the main experiments described above. At present, our major need is for tumors with which experiments like the above can be repeated but with certain variations, especially a reversal of the interstrain relationships. We are studying several tumors in a search for suitable ones. Experiments designed to throw some light on the mechanism of "maternal tolerance" will be continued.

Part B Included: Yes X No

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Breyere, E. J., and Barrett, M. K.: "Tolerance" in postpartum female mice induced by strain-specific matings. J. Nat. Cancer Inst. 24: 699-705, 1960.

Brevere, E. J., and Barrett, M. K.: A strain-specific influence of parity on resistance to homografts. Ann. N. Y. Acad. Sc. 87: 112-117, 1960.

Breyere, E. J., and Barrett, M. K.: Prolonged survival of skin homografts in parous female mice. J. Nat. Cancer Inst. (In press).

Honors and Awards relating to this project:

Dr. M. K. Barrett - Elected to Washington Academy of Sciences.

Serial No. NCI-419
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies of the presence of antibody to Polyoma Virus in mice.

Principal Investigator: Malcolm B. Melroy

Other Investigators: None

Cooperating Units: M. Fink (NCI-415)

Man Years (Calendar year 1960):
Total: 1 1/3
Professional: 1
Other: 1/3

Project Description:

Objectives: To determine incidence of polyoma infection in individual mouse colonies in Laboratory of Biology.

To test groups of mice prior to inoculation with experimental materials to rule out any possibility of confusion with polyoma infection.

To test experimental mice after the introduction of materials which might have contained polyoma virus.

Methods: Mice are bled by puncture of the inner canthus of the eye. The blood is allowed to clot and the serum removed for the serological test. The antigen used for this test is obtained from the tissue cultures of polyoma virus. The hemagglutination inhibition test is used according to the Rowe, Hartley and Huebner method.

Major Findings: The General Biology, Virus Oncology, Tissue Culture and Cellular Biology Sections have had over

four hundred mice tested for polyoma virus. The colonies of mice of the different sections have been free of polyoma virus. The only presence of the virus was in the inoculated mice with material suspected of the virus.

In the Leukemia Studies Section over six hundred mice have been tested for polyoma virus. Many of the sera were tested both quantitatively and qualitatively for the virus so the investigator may have some knowledge of the nature of the virus in relation to the tumors in the host. Many tissue cultures of polyoma virus have been tested for their potency.

Mice tested for polyoma virus in the Laboratory according to Sections:

General Biology	110
Virus Oncology	336
Tissue Culture	99
Cellular Biology	21
Leukemia Studies	617
Total	1183

Significance to bio-medical research and the program of the Institute: Because of the prevalence of polyoma virus in the environment it is necessary to know that mice used in experimental cancer research are polyoma free. Also if polyoma infection is found in a previously "clean" colony, steps can be taken to prevent spread.

Proposed course of project:

1. Continuation of studies on incidence of polyoma infection in different mouse colonies.

2. Continuation of screening experimental mice prior to their use in cancer research.

Polyoma virus is highly resistant to various environmental influences. The loss in hemagglutination or infectivity titer after exposure to various disinfectants, temperatures, freezing, thawing and other environmental conditions are to be studied.

Part B Included Yes No X

Serial No.NCI-421
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Genetic studies in mice. A. Tumors in mice

Principal Investigator: Margaret K. Deringer

Other Investigators: W. E. Heston, M. K. Barrett

Cooperating Units: NCI-417 (Heston); NCI-418 (Barrett)

Man Years (Calendar year 1960):
Total: 2
Professional: 1
Other: 1

Project Description:

A. Tumors in mice.

1. Occurrence of mammary tumors in agent-free DBA/2eB mice.

Objectives: Strain DBA/2eB, a substrain of strain DBA/2, was produced by the transfer of fertilized ova from strain DBA/2 to C57BL. The development of mammary tumors and of other tumors is being studied in groups of virgin, breeding, and force-bred females, and in males treated with stilbestrol.

Methods Employed: A group of DBA/2e females was kept as virgins and another group is force-bred. The force-bred females are isolated when pregnant. After the birth of the litter, the young are discarded, and the mothers are returned to the breeding cages at once. This procedure is continued throughout the breeding period of the females. Cholesterol pellets, weighing 6 to 8 mg. and containing 10% diethylstilbestrol,

were implanted subcutaneously in the right axilla of DBA/2e males that were one to two months of age. The breeding females were those that were used to maintain the colony of DBA/2e mice. The various groups of mice were observed at regular intervals for the development of mammary tumors and of other lesions.

Major Findings: About one-fourth of the force-bred females of strain DBA/2e are still alive though only a few females are still breeding and producing young. The incidence of mammary tumors is considerably lower in agent-free DBA/2e mice than in DBA/2 mice. In the colony of strain DBA/2 (with the agent), 85 of 110 breeding females (77.3%) have developed mammary tumors at an average age of 14.9 months. Mammary tumors have developed in one strain DBA/2e breeding female (at 20 months of age), in one DBA/2e male (treated with stilbestrol) at 12 months of age, in two virgin females at 22 and 24 months of age, and in 15 of 46 force-bred females (32.6%) at an average age of 16.5 months.

Significance to the program of the Institute: This experiment will supply information concerning the development of mammary and of other types of tumors in a strain of mice deprived of the mammary tumor agent by the procedure of transferring ova from an inbred strain of mice carrying the agent to another strain without the agent. The results will reflect on the interpretation of the relative effects of the genetic constitution, the mammary tumor agent, and the hormonal influence on the development of mammary tumors.

Proposed course of the project: Further data on the development of tumors in the force-bred females will be collected. When autopsies have been performed on the remaining force-bred females, the information obtained will be combined with that from the virgin females and from the females of the breeding colony and the material will be prepared for publication. The strain of mice will be continued and the development of mammary tumors will be studied in subsequent generations.

2. Production of tumors of the skin

Objectives: To attempt to produce tumors of the skin in strain HR mice by painting with urethan. Since it has been stated that urethan is "non-carcinogenic for skin epithelium in the mouse" it seemed worthwhile to treat mice of strain HR, that are highly susceptible to the development of induced tumors of the skin, with this compound.

Methods Employed: A 40% solution of urethan in ethylene glycol is painted on the skin of haired and hairless animals of both sexes

of the HR strain. Other groups of males and females are painted with ethylene glycol alone. Twice weekly paintings were started when the animals were 2 1/2 to 3 1/2 months of age and are being continued throughout the lifetime of the animals.

Major Findings: Data obtained indicate that urethan is probably not carcinogenic for the skin of strain HR mice. However, tumors of the skin have been observed in 17 of 26 hairless females, in 1 of 17 haired females, and in 7 of 23 hairless males that have been painted with urethan in ethylene glycol. Nine of 19 hairless females and 4 of 12 hairless males, painted with ethylene glycol alone, developed tumors of the skin. None of the tumors arose in the painted area.

Painting the skin with urethan does induce pulmonary tumors and hemorrhagic lesions of the liver in haired and hairless animals of strain HR. Pulmonary tumors have developed in 16 of 26 hairless females, in 16 of 17 haired females, in 13 of 23 hairless males, and in 12 of 17 haired males that were painted with urethan in ethylene glycol. They developed in 2 of 19 hairless females, in 2 of 17 haired females, and in 1 of 12 haired males that were painted with ethylene glycol. Hemorrhagic lesions of the liver have developed in 18 of 26 hairless females, in 15 of 17 haired females, in 17 of 23 hairless males, and in 14 of 17 haired males that were painted with urethan in ethylene glycol; and in 2 of 19 hairless females, 2 of 17 haired females, and in 1 of 12 haired males that were painted with ethylene glycol. Most of the lesions in the liver can be classified as hemangioendotheliomas while a few appear to be blood-filled spaces. Hemorrhagic lesions of the liver in 5 animals have been transplanted. Of these 4 grew, one of which has been transplanted 8 times and 2 others for the second time.

Significance to the program of the Institute: This study will add information concerning the influence of genetic constitution on the development of tumors. The effect of a carcinogen on the development of a particular type of tumor, to which the skin of animals is susceptible, will be determined.

Proposed course of the project: When the small group of animals, presently being painted, have been autopsied, the data obtained on the development of various types of tumors will be prepared for publication.

3. Influence of the lethal-yellow ($A^{y}a$) gene on the development of leukemia in mice.

Objectives: To determine the relationship of the lethal-yellow gene (known to increase body size) and the occurrence of leukemia in treated and untreated animals (in cooperation with Dr. Heston).

Methods Employed: Crosses have been made between strain DBA/2, a strain susceptible to the development of leukemia and strain YBR, a strain carrying the lethal-yellow gene. A group of F_1 mice were painted 3 times a week for 7 weeks (7 different sites were used) with an 0.2% solution of 3-methylcholanthrene in ether. A second group of F_1 animals has been maintained without treatment. The two groups are observed at regular intervals for the development of leukemia and of other types of lesions.

Many of the (DBA/2 x YBR) F_1 females have developed mammary tumors at an early age thus eliminating a large number of females from the study. In order to study more adequately the effect of the lethal-yellow gene on the development of leukemia, a second F_1 cross between strains YBR and AKR (a strain highly susceptible to the development of leukemia) has been made. A total of 200 females and 200 males have been produced (all of these animals are still alive and are less than 7 months of age).

Major Findings: Mammary tumors have developed in both the yellow and brown (DBA/2 x YBR) F_1 females of the treated and untreated groups. Forty-three of 49 yellow females (87.8%) of the treated group developed mammary tumors at 9.6 months of age as compared with 33 of 47 (70.2%) of the untreated group at 14.5 months of age. Eleven of 17 brown females (64.7%) of the treated group developed mammary tumors at 10.7 months of age as compared with 9 of 23 (39.1%) of the untreated group. These results are in accord with the observation that both segregants develop mammary tumors but that the yellow segregants develop them earlier.

At the present time no effect of the lethal-yellow gene on the development of leukemia in (DBA/2 x YBR) F_1 animals appears to have been demonstrated. Only a small number of animals with reticular lesions has been observed.

Hepatomas have been observed in 27 of 47 untreated yellow females and 6 of 23 untreated brown females. They have been observed in 25 of 25 untreated yellow males and in 14 of 16 untreated brown males. Multiple hepatomas have usually been seen. Occasionally an unusual liver tumor has developed. This type of tumor has been compared by pathologists to a type seen in human infants.

Tumors of the skin have been observed in a number of the painted animals despite the fact that the carcinogen was painted on 7 different sites in rotation. These tumors have occurred more frequently in the yellow than in the brown females and about equally in the yellow and brown males of the treated groups.

Significance to the program of the Institute: This experiment will demonstrate the role of the lethal-yellow gene in the development of leukemia in mice.

Proposed course of the project: All animals of the three experimental groups will continue to be observed at regular intervals for the development of tumors and of other lesions.

4. Amyloid in the organs of strain BL mice.

Objectives: The observation that the corpora lutea of the ovaries of strain BL breeding females were often heavily infiltrated with amyloid prompted the present study of the distribution of amyloid in other organs of strain BL mice.

Methods Employed: Groups of male and female mice of strain BL were set aside and are autopsied at 15 months of age. Sections of various organs are made and data on the distribution of amyloid are collected.

Major Findings: In female mice of strain BL, amyloid has been found in the ovaries, duodenum, and ileum of all animals studied. Amyloid was observed in the spleen in about 1/2 of the females, and in the uterus and kidney in about 1/3. It was seen occasionally in the liver and in the vagina. In males of strain BL, amyloid was observed in the duodenum and ileum of all animals, in the spleen of 1/2, and in the liver and kidney of about 1/5 of the animals studied.

An incidental finding in this study is the persistence of amyloid in transplanted ovarian tissue. One-half of each of the ovaries of 4 females (autopsied at 15 months of age) were transplanted into the right axilla of BL females. When sections were made of the transplanted tissue 6 months later, amyloid was still present in the ovarian tissue. Histologic sections of the other pieces of each ovary had revealed the presence of amyloid at autopsy.

Significance to the program of the Institute: This study will provide more information concerning the characteristics of one of the highly inbred strains maintained at the Institute.

Proposed course of the project: Data on the distribution of amyloid will be secured for a representative group of animals and the material obtained will be prepared for publication.

5. Occurrence of mammary tumors in agent-free RIII/eB mice.

Objectives: To produce an agent-free subline of strain RIII, a

strain with a high incidence of spontaneous mammary tumors and to study the incidence of mammary tumors in various groups of animals of such a subline.

Methods Employed: A colony of RIII mice has been established from a breeding litter obtained from Dr. H. B. Andervont in December, 1959. Six females of the line, that has been established, have developed mammary tumors at an average age of 7 months. Fertilized ova will be transferred from the females of this high-mammary-tumor line to females of a low-mammary-tumor line and a colony of RIIIeB mice established.

Major Findings: None to date.

Significance to the program of the Institute: This study will supply data about the development of mammary and of other types of tumors in a subline of mice deprived of the mammary tumor agent by the process of transferring ova from an inbred strain of mice carrying the agent to another strain without the agent. The results will be of importance in the study of the three factors influencing the development of mammary tumors.

Proposed course of the project: Strain RIII and strain C57BL females will be observed for the presence of vaginal plugs following their return to the breeding cages at the time their litters are weaned. When suitable animals have been found ova transfers will be made with the aim of securing a breeding pair in order to start a RIIIeB subline.

6. Production of leukemia with urethan.

Objectives: To attempt to produce leukemia in strain DBA/2eB mice by treatment with urethan. It has been demonstrated that urethan induces various types of tumors and that it augments the production of leukemia by x-rays, estrogenic hormones, and methylcholanthrene.

Methods Employed: A group of strain DBA/2e mice will be injected intraperitoneally with an aqueous solution of urethan, groups will be painted with a solution of urethan in ethylene glycol, a group will be painted with ethylene glycol alone, and a group will be maintained without treatment.

Major Findings: None.

Significance to the program of the Institute: If successful this experiment will add another type of tumor to the list of those induced by urethan, originally thought to produce only pulmonary tumors.

Proposed course of the project: Strain DBA/2e females are being set aside and when sufficient numbers have been obtained, treatment with urethan will begin.

7. Immunogenetic experiments.

Objectives: To produce special types of animals for collaborative experiments with Dr. M. K. Barrett (see Dr. Barrett's report for details).

Part B Included Yes X No

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Heston, W. E., Vlahakis, G., and Deringer, M. K.:
High incidence of spontaneous hepatomas and increase of this incidence with urethan in C3H, C3Hf, and C3He male mice. J. Nat. Cancer Inst. 24: 425-435, 1960.

Heston, W. E., Vlahakis, G., and Deringer, M. K.:
Delayed effect of genetic segregation on the transmission of the mammary tumor agent in mice. J. Nat. Cancer Inst. 24: 721-731, 1960.

Honors and Awards relating to this project:

Elected to membership in the Washington Academy of Sciences, June, 1960.

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Serial No. NCI 427
1. (Laboratory of Biology)
2. (Leukemia-Studies Section)
3. (Bethesda 14, Md.)

Part A.

Studies on the etiology and therapy of experimental lymphomas

L. W. Law

Man years (calendar year 1960)
Total: 5
Professional: L. W. Law, 1
Other: 4 (3, 1/2, 1/2)

A. Role of several variables in X-ray induced and spontaneous leukoses

1. Chimeras developed in C3Hf/$_{Lw}$ and C3Hf/$_{Bi}$ strain mice by the intravenous introduction of AKR bone marrow into neonatal mice (<12 hrs. of age) have developed lymphocytic and other neoplasms of reticular tissues at the same frequency and latent period as non-chimeric controls. Introduction of AKR thymic tissue into such chimeras at 1-2 months of age has resulted in an increased frequency of lymphocytic neoplasms in the C3Hf/$_{Lw}$ strain from 10% to 30%; mean age at death was at 13.3 months. The frequency and distribution of morphologic forms of neoplasm (lymphocytic, granulocytic, plasma cell and monocytic) in the C3Hf/$_{Bi}$ strain was 23.2% and mean age at death 14.3 months, similar to controls. Thymic grafts persisted in the chimeric animals and were recoverable but relatively few were involved in the leukemic process. Successful transplantations of lymphocytic neoplasms were achieved only in the host (C3Hf/$_{Lw}$) strain and not in AKR mice. These results are at variance with those reported by Miller who used thymectomized chimeras; the presence of C3H thymus may indeed exert an inhibitory influence on the process of leukemogenesis.

2. An attempt has been made, using the Moloney technique, and in collaboration with Dr. Moloney to isolate a

Part A.

cell-free material from C3Hf/Fg leukemias. This subline has a high frequency of leukoses, 80% in females, 50% in males, with distribution of forms among lymphocytes, granulocytes, plasma cells and monocytes. Few develop thymic lymphomas and thymectomy is relatively ineffective in changing the frequency of morphologic types. Injections of cell-free materials into C3Hf/Fg, C3Hf/Bi, (C3HxAKR)F1 and DBA/2 mice during the neonatal period have not significantly altered the frequency of leukoses within an 8 month observation period.

3. X-radiation and induction of experimental leukoses

a). 100r and 150r total body irradiation given to pregnant C57BL/Ka strain mice at 10 1/2 days and at a later time, 15 to 18 days, is ineffective as an in utero leukemogen despite the fact that this same total dose is leukemogenic for 24 hour and 1 month-old C57BL/Ka mice. This striking insensitivity of fetal tissues, observed also by Upton (300r in RFM mice) deserves further study at periods of fetal development specifically when yolk-sac, liver and bone marrow function as hematopoietic tissues.

b). Much emphasis has been placed in the past on the role of total body, fractionated X-radiation and on the effect of shielding of hematopoietic tissues on the induction of thymic lymphomas. We have observed that total body radiation (168r x X4) is effective in thymectomized C57BL/Ka mice, reticulum cell sarcomas (Type A and B) and granulocytic neoplasms de veloping in nearly 40% of mice beyond 1 year of age, compared with 10-20% in unirradiated controls. Shielding of a thigh, in contraxt to this influence on thymic lymphoma induction, has been observed to be without effect on the induction of these later appearing forms of leukoses.

c). Interesting differences in response to fractionated, whole-body X-radiation have been observed among

Part A.

three C3H sublines: C3Hf/$_{Bi}$, C3Hf/$_{Lw}$ and C3Hf/$_{Fg}$. Rapid appearance and high frequency (60%) of thymic lymphomas appear in the Bittner substrain (C3Hf/$_{Bi}$) particularly in the substrain kept in our laboratories for 5 years, but a significantly lower frequency in a subline obtained directly from Bittner and X-rayed here. A poor response to X-radiation has been observed in the C3Hf/$_{Lw}$ subline (20%) with few thymic types. Thymic lymphomas appear early in the high leukemic C3Hf/$_{Fg}$ subline and these are prevented by isologous bone marrow, but a striking reduction in frequency occurs of the typical neoplasms observed to occur spontaneously. Thymectomy also prevents the effects of X-radiation on the induction of the thymic lymphomas in the C3Hf/$_{Fg}$ subline.

d). Characterization of the inhibitory effect of bone marrow upon the induction of leukoses by X-rays in C57BL and BAF$_1$ hybrids is continuing:

1. Thymic grafts introduced 21 days following bone marrow remain non-responsive. This period will be extended to 6 months.

2. Allelographs of bone marrow (H-2^d rather than H-2^b) are ineffective in presenting X-ray induction whereas substitutions at other loci (H-1 and H-3 and others), keeping H-2 the same (H-2^b) prevents thymic lymphomas. These data are now completed.

B. Cell-free and tissue culture agents in the induction of lymphomas and other neoplasms

1. A study attempting to correlate number of particles, hemagglutination titer (HA), mouse infectivity and tissue culture infectivity has been continued in collaboration with Drs. Kahler and Lloyd (NCI)/Drs. Rowe and Hartley (LID) and Dr. Dawe (LP) and is near completion. The results are reported more fully in the report of Dr. Kahler. In this study using 4 different strains (?) of parotid tumor (polyoma) virus and (C3HxAKR)F$_2$ test mice certain preliminary

Part A.

observations recorded are:

a) A highly virulent strain required only 20-30 particles for tumor formation; another required more than 10^6 particles.

b) Titration of the highly virulent virus strain resulted not only in a reduction of oncogenicity but also in a change in the pattern of tumor induction.

c) There were, in certain instances, a lack of correlation of oncogenicity and the other parameters studied.

2. One study bearing on the basic nature of viral selectivity concerns the pronounced resistance of C57BL/Ka strain mice to the highly virulent thymotrophic P-388-D_1 passaged polyoma. After an observation period of 6 months 92% of C3Hf/Bi mice have developed thymic epithelial (and other) tumors while 0% C57BL/Ka mice have developed tumors. Only 10% of F_1 mice (BxC3H and C3HxB) have been tumorous.

a) Backcrosses of F_1 mice to the susceptible (C3H) strain and to the resistant (B) strain have been made to further understand the possible genetic basis of resistance,

b) A study of the pattern of polyoma growth in the neonatal period of B and C3H mice has been initiated with Drs. Rowe and Hartley (LID),

c) It is planned to study the effect of polyoma upon intact fragments of salivary gland tissue of the newborn C57BL/Ka mouse in vitro with Dr. Dawe (LP). The morphologic changes characteristic of the neoplastic transformation of C3H strain tissues have been documented with this system.

3. The occasion was presented to study the influence of tissue culture medium on infectivity and oncogenic properties of the P-388 D_1 passaged thymotropic strain of polyoma.

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At the time this strain was introduced into P-388 cells in 40% human serum medium, it was also introduced into P-388 cells adapted to grow on a milk medium. After 15 passages, these 2 substrains were compared in regard to infectivity and oncogenic potentialities. The substrain grown on P-388 cells in milk medium retained high infectivity for mouse (HA) and for tissue culture (CPE) but were nearly non-oncogenic, whereas the human serum passaged material, with a reduced infectivity HA and CPE, remained highly oncogenic, 93% tumors vs. 9%. The virus grown in milk adapted P-388 cells, however, retained its oncogenicity for hamsters. (Collaborative work with Drs. Rabson and Dawe , (LP, NCI).

4. Transfer of polyoma infected fragments of salivary gland tissues after 30-50 days as explants on a sponge-matrix system have been made in vivo to X-rayed adults and to mice during the neonatal period. Several neoplasms have developed from such explants. Two, fibrosarcomatous in character and one, epithelial in character have been studied. Both fibrosarcomas appear to be free of virus but the epithelial tumor still produces virus. Exhaustive attempts to demonstrate virus in one of the fibrosarcomas have been made and include CPE tests in tissue culture, MAP tests in NIH colony mice, development of hemagglutinins (HA) blind passage in tissue culture, etc. No virus has been detected in material from the original through 8 transfers of tissue in HI negative mice. The results are similar to those reported by Dulbecco and Sanford (LB, NCI) in their studies of fibrosarcoma development in vitro. It is difficult at the moment to determine whether or not this represents a phenomenon analogous to lysogeny in bacteria.

5. Studies have been completed concerning other phases of the polyoma problem:

a) X-radiation (300r, whole-body) preceding introduction of polyoma into adult $C3Hf/_{Bi}$ mice renders these susceptible to virus; 38% and 43% adults presented single or bilateral

Part A.

parotid gland tumors following intravenous introduction of thymotropic polyoma virus. The response therefore differs strikingly from that of the same strains infected in the neonatal period. Thymectomy of adults had no influence on the carcinogenic response and virus or X-rays alone were ineffective. X-radiation plus natural infection (at 3 to 5 months of age) by polyoma did not induce neoplasms characteristic of this virus.

b) A peculiar property of epithelial tumors of the thymus in C3Hf/Bi , C3Hf/$_{Lw}$ and in (C3HxAKR)F_1 mice is their inability to "take" and grow progressively in mice of the isologous strain, either in young mice or in X-rayed adults. Of 28 individual neoplasms transferred by various means and methods to 52 litters (347 mice) only 3 tumors have shown progressive growth and continued growth upon subsequent transfers. One of these interestingly, which arose in a ♀ C3Hf/$_{Bi}$ showed a striking hormone-dependency through the early transfer generations, growing only in females or in males treated with estrogen. Growth was prevented in castrated females treated with testosterone.

6. In collaboration with Dr. John Moloney (NCI), a study of possible maternal and paternal transmission of Moloney leukemia agent is being carried out using 2 low leukemic strains of mice C3Hf/$_{Lw}$ (low sensitivity) and C3Hf/$_{Bi}$ (high sensitivity), a high-leukemic (late appearing leukemia) strain, C3Hf/$_{Fg}$ and a highly susceptible strain RFM. Transfer of virus (or leukemic cells) has been observed in RFM and C3Hf/$_{Bi}$ mice to offspring from preleukemic or leukemic mothers. To date leukemia has not been observed in offspring obtained from infected fathers or in offspring born of infected mothers but foster-nursed. Many of the mice (males and females alike) appear sterile following introduction of the Moloney agent. Studies have been commenced to determine, using another source of agent, whether this is repeatable and if so what determines the sterility.

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7. A comparative morphologic study of leukemic mice resulting from the Gross agent, the Moloney agent, spontaneous AKR, X-ray induced leukemia from C57BL/Ka mice, and leukemia from C3Hf/Fg mice is being done in collaboration with Dr. Albert Dalton (NCI).

C. Studies of mechanisms involved in inhibition of leukemic cell growth

1. The collaborative study with Dr. Wallace Brockman, Southern Research Institute and Dr. E. Anderson, this Section, is continuing. Mechanism of resistance studies to 5-fluoropyrimidines employing several P-815 and L1210 lines have yielded interesting results. Both neoplasms resistant to FU have a decreased capacity (or decreased rate) of nucleotide formation. Phosphorylase and kinase activities are found in R lines in contrast to findings by Reichart. Enzyme studies will be continued and attempts to develop resistance to FU in P-815 *in vitro* will be commenced.

2. Collaborative study is underway with Dr. Milton Silverman (NIAMD) in examination and determination of folate derivatives in leukemic cell lines. Chromatography and microbiological assay in P-815 (mast cell neoplasm) and 70429 (plasma cell neoplasm) have revealed the presence of principally N^{10} formyl-THFA. Interestingly, no polyglutamyl derivatives could be detected. This work is continuing in a study of other leukemic lines, those resistant to certain drugs and of comparable normal tissues.

3. Collateral sensitivity studies.

a). Cyclophosphamide (cytoxan) has been found to exert a striking antileukemic effect against both P-815 and L1210, particularly the former. The development of resistance to 5-fluoropyrimidines apparently has changed the sensitivity to cytoxan. All R lines (FO, FU and FUR) were found to be more sensitive than the S (sensitive) parental line. At optimal

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dosage levels, complete regressions of established tumors in both P-815 and L1210 have been observed (collaborative study with Dr. M. Lane).

b). A triply-resistant subline of the lymphocytic neoplasm L1210 has been developed to the agents 6-mercaptopurine, 5-fluorouracil and Amethopterin (L1210/$_{MP-FU-AM}$). Growth rate and strain sensitivity have been altered in this subline. Experiments are now in progress to study the comparative response in this and the parental line (L1210/$_{S}$) to some of the newer effective drugs particularly Glyoxal, bis (amidinohydrazine) sulfate, Diozouracil, 1-methyl-1 nitroso urea, Pyruvaldehyde and azaserine derivatives.

4. The plasma cell neoplasm 70429 which is sensitive to 6-azauridine has been used to develop resistance to this compound. A stable, heritable subline (also stable through serial cloning) to 6-azauridine was developed after 8 transfers in C3H mice given 6-azauridine *per os*. This subline is cross resistant to 6-azauracil and a 6-azauracil-resistant subline of 70429 is cross-resistant to 6-azauridine. The possibilities of a major involvement of orotic acid decarboxylase in resistance is being investigated by Drs. Fallon and Frei (General Medicine Branch).

D. Studies in mammalian somatic genetics and carcinogenesis

Dr. Robert Roosa has terminated his NIH post-doctoral fellowship. Two interesting findings are as follows:

1. The P-388 lymphocytic neoplasm, established as a continuous cell culture was used to develop resistance to high levels of 8-azaguanine. Several sudden and large steps were isolated and cloned. The P-388/$_{AG}$ (resistant to azaguanine) cells appear to have lost guanylic and inosinic acid pyrophosphorylase activity, resembling in this respect the "deletions" observed in certain bacterial cultures through use of antipurines as inhibitors.

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2. Preliminary date now obtained in three separate instances, point to the possibility that stable, DNA-induced transformations in mammalian cells may be demonstrable. P-388 sensitive cells incubated in vitro with DNA prepared from cells with a high level of 8-azaguanine resistance (P-388/Agr-2) have developed resistant colonies at a frequency 20-40 times that observed in sensitive control cultures. It is proposed to continue this work here with Dr. Ray Bradley (Anna Fuller Fund Fellow) and independently by Dr. Roosa at Wistar Institute. Effects of concentration of DNA, of varying incubation periods, of cloning resistant colonies following DNA treatment to determine their heritability, etc. are continuing.

Dr. R. T. Bradley joined this section in January as an Anna Fuller Fund Fellow. A technique has been developed which allows one to perfuse substances into the extra cellular fluid environments of a designated piece of tissue or population of cells in normal unrestrained experimental animals for periods of up to several weeks.

This technique has been used to perfuse substances continuously into millipore diffusion chambers containing P-388 culture cells or L1210 cells and implanted in the peritoneal cavities of mice.

When such chambers were perfused with Eagle's basal medium (with pyruvate, serine and glutamine) for 7 days at the rate of 1 ml. per day, the approximate generation time was 12 hours compared with 24-32 hours in control non-perfused chambers.

However, the effect varied with the batch of medium supplied. Thus in an attempt to trace the origin of the effect various components of the medium were tested with the following results:

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The growth rates are expressed as a percent of that in control chambers

		Per Cent
1.	Locke's solution	70
2.	Earle's salts	43
3.	ES + amino acids (AA)	25
4.	ES + AA + vitamins (vit)	27
5.	ES + AA + vit + pyruvate) serine) glutamine)	17

These results give no clue to the origin of the stimulatory effect observed, hence other combinations are being tested

In the course of investigating the use of the continuous injection technique in connection with perfusion of thymus tissue grafted under the kidney capsule of month old, thymectomized and fractionally irradiated C57BL/$_{Ka}$ mice, preliminary work has indicated that isologous thymus grafts in this situation do not give rise to lymphoid tumors. No thymic tumors have been observed at 9 months following grafting whereas 21 of 31 (68%) thymic grafts in the subcutaneous tissues of the right axilla have appeared at an average age of 5-6 months (3.6 months following grafting).

The continuous perfusion technique is also being used in collaboration with other groups of research workers at NIH.

In collaboration with Dr. R. Roosa (Wistar Institute) attempts have been made to demonstrate a DNA induced transformation, with respect to drug resistance in mammalian cells in vitro. Two experiments have yielded apparently positive results with parental P-388 incubated with DNA extracted from a subline of P-388 which is resistant to 8-azaguanine. These esperiments are being continued. (See above).

Part B Included Yes X No

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Part B.

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Law, L. W., Rowe, W. P., and Hartley, J. W. Studies of Mouse Polyoma Virus Infection V. Relation of Virus Infection to Lymphocytic Neoplasms of the Mouse. J. Exper. Medicine, 111(4): 517, (1960)

Anderson, E. P., and Law, L. W. Biochemistry of Cancer. Annual Review of Biochemistry, Vol. 29: 577, (1960)

Law, L. W. "Radiation Carcinogenesis" In Advances in Biol. and Med. Physics. Vol. VII; 295 (1960) Academic Press. N.Y.

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Part A.

Studies on: (1) Pathogenesis and pathophysiology of reticular neoplasms in mice and, (2) genetics of somatic cells

Michael Potter

Man years (calendar year 1960)
Total: 4
Professional: Potter - 1
Other: 3

A. Plasma-cell neoplasia in mice

1 a.) Pathogenesis - induction of plasma-cell neoplasms

Very little is known about the pathogenesis of multiple myeloma, a disseminated plasma-cell neoplasia of man. The susceptibility of Strain BALB/C mice for the development of intraperitoneal plasma-cell neoplasia (evoked by a variety of means) has provided the first good system for the experimental study of this disease. Information obtained may provide guides for revealing specific environmental factors in the pathogenesis of multiple myeloma. Since the tissue involved is the mesenchymal differentiation to plasma cells (the process whereby certain antibodies are elaborated) this study is related intimately to immunology and the biology of protein synthesis. The inbred mouse has been a much neglected experimental animal by immunologists, while cancer researchers and geneticists have extensively used this animal. The infusion of new immunologic information and techniques will greatly expand our current knowledge of carcinogenesis and mammalian genetics. The study of plasma-cell neoplasia and indeed other reticular neoplasms is a meeting ground for many of the related areas.

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It was found that BALB/C mice, given single intraperitoneal injections (0.3-0,5 ml) of adjuvant-staphylococcus mixtures (IS) (1 part incomplete Freund's adjuvants plus 1 part of concentrate of a heat-killed staphylococcus broth culture) developed plasma-cell neoplasms. The neoplasms developed in the lipo granulomatous tissue that formed in all mice following injection of the adjuvants.

The immediate objectives of the studies in progress are: 1) to find a means for inducing plasma-cell neoplasms with high frequency; 2) to introduce a specific immunization procedure during the development of the plasma-cell neoplasm for purposes of studying the functional properties of myeloma globulins (are myeloma globulins antibodies?); 3) to provide a continuing source of new transplantable plasma-cell neoplasms for a variety of purposes; 4) to give new information and a new approach to the study of carcinogenesis by examining the development of neoplasia in a highly functional cell system.

The experiments currently in progress are listed in Table 1. Thirty-one plasma cell neoplasms have been found thus far in these experimental animals. The minimum period for development of a plasma cell neoplasm is 5 months after the intraperitoneal injection of IS. Plasma cell neoplasms have been found at least as long as 21 months after the i.p. injection. IS is a complex mixture, its ingredients have been studied for activity. At this time 4 plasma-cell neoplasms have been found in a group of 40 mice injected with 0.4 ml of paraffin oil (Bayol F) alone. It had been hoped that the injection of the paraffin oil alone would provide a control procedure. A vegetable oil (corn oil) is currently being used for this purpose. U.S.P. grades of paraffin oil will be injected in forthcoming experiments. (A current clinical procedure used by some allergists has employed the intra-muscular injection of mineral oil). Other substances to be injected in BALB/C mice will be croton oil, colloidal methylcholanthrene, B.C.G. Specific immunization procedures concurrent to

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TABLE 1

Exp.	Procedure				Dead		
	Initial injection	IP. ml.	Strain	Other	Total	Other	Plasma Cell Neop.
1	IS	0.3	C	-	24	21	3
	IS	0.3	C	MCA sk. pt.	31	6	6
2	IS	0.5	C	-	18	1	3
	IS	(0.5x2)	C	-	19	4	7
3	IS	0.4	C		32	2	0
	IS	0.4	C	imm. horses	35	2	2
	IS	0.4	C	imm. horses	28	2	1
4	IS	0.4	C	-	38	1	1
	IS	0.4	C	-	31	2	2
	IS	0.4	DBA	-	41	2	0
	I	0.4	C	-	40	1	4
	M.O.	0.4	C	-	40	2	0
5	I	0.2x2	C	-	32	0	0
	I	0.2x2	C	imm. Homarus, s.	40	0	0
6	IS	.4	C	-	32	0	1
	IS	.4	C	imm. Limulus s.	32	3	2
7	IS	.4	C	-	46	0	0
	IS ingredients not mixed				56	1	0
8	IS	0.4	C		24	0	0
	IS	0.4	C3H		40	0	0
	IS	0.4	C57BL		40	0	0
	IS	0.2	C	(S.C.)	40	0	0
9	M.O.	0.5	C		32	0	0
	M.O.	0.5x2	C		32	0	0
	C.O.	0.5	C		40	0	0
	Lockes	0.5	C		40	0	0
10	M.O.	0.5	C		32	0	0
	Silica	10 mgm.	C	I.V.	32	0	0
	Silica	10 mgm.	C		24	0	0
	Silica	10 mgm.	C		24	0	0

(See Footnotes, next page)

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Footnotes to Table 1:

I = Incomplete Freunds
S = Heat-killed staphylococcus culture
IS = I + S mixed
M.O. = Bayol F
Silica = Colloidal suspension 5μ silica particles
imm. = Immunization
s. = serum
C.O. = Corn oil (Mazola)

Part A.

injection of I.F. have used, Homarus and Limulus (rich in hemocyanin - a good antigen for mice) and Horse Serum. Experiments using Vi antigen (to be done in collaboration with Dr. M. Landy, Lab. of Chem. Pharm.) as an antigen are in progress. Strains other than BALB/C have been used, including DBA f/2. C57BL and C3H/He. At this time plasma-cell neoplasms have not been found in these strains.

1 b.) Histologic and cytologic study of plasma-cell neoplasms.

In collaboration with Dr. Ross C. MacCardle of the Laboratory of Pathology, NCI, a histologic and cytologic study of primary plasma cell neoplasms is nearing completion.

1 c.) Transplantation studies (parallel line transplantation study)

Growing transplants of plasma-cell neoplasms produce quantities of serum myeloma globulin in new recipients. The myeloma globulins are a heterogeneous group of related proteins. Specific myeloma globulins are produced by different tumors. When a tumor is established in transplant, the specific myeloma globulin production is constant for that tumor and remains qualitatively the same throughout the transplant history. Very few studies have been done on establishing different protein-producing lines from one original host. One such case has been found. The primary host of Adj-PC 6, gave rise to two different, stable transplant lines Adj-PC 6 A, and Adj-PC 6 C.

The primary peritoneal plasma-cell neoplasm appears to arise multifocally for the reactive mesentery contains numerous nodules of seemingly incipient plasma-cell neoplasms. In early cases the nodules are separated and permit transplantation of wedges of mesentery containing the nodules. In late cases, overgrowth by a rapidly progressing neoplasm obscures this important detail. Using specific protein production as the indicator, each new case is being selected for use in this study.

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The ideal means for successfully establishing transplants has not been found. Many routes have been explored.

The purpose of the study is to further localize the time and site of origin of the carcinogenetic event. Does the plasma-cell neoplasm begin as a disorder of the mesenchyme which is revealed only when the differentiation to plasma-cell takes place?Using this biologic system it is hoped further information on mechanism of carcinogenesis will be gained.

2. Studies on myeloma globulins in mice

Over 24 different plasma-cell neoplasms are maintained in transplant, 20 of the neoplasms are of strain BALB/C origin, a list of these is given in Table 2, which also includes specific characteristics of the neoplasms. The parallel line study was described in 1 c. The other categories are individual studies in progress.

2 a.) Immunoelectrophoresis

The myeloma globulins have been characterized by agar-gel immunoelectrophoresis using anti sera from rabbits immunized with washed microsomes (Pw fractions). These fractions have been prepared by Dr. E. L. Kuff of the Laboratory of Biochemistry, NCI.

The microsomes contain the antigenic determinents of the serum protein myeloma globulins. Similar antigenic determinates are found on normal immuno globulins such as gamma-2 globulin (SI) and a certain Beta globulin (SII). Three anti microsome antibodies (Pw anti sera) have been studied.

Tumor Source	Precipitates formed with normal serum
MPC 1	SII
MPC 2	SI SII
Adj-PC 5	SI

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TABLE 2

Neoplasm	Immuno-electrophoretic characterization	e.m. Mobility	Precipitate at low ionic strenth	Imm.	Pw anti serum made	Parallel lines
MPC 1	SII	alpha	-		+	
2	SI, SII (BJ)	beta	-		+	
3	SII	beta	-			
4	SII	alpha	-			
LPC 1	SI	gamma	+		+	
AdjPC 2	SII	beta				
" 5	SI	gamma	-		+	
" 6A	SI	?	++		+	+
" 6C	SI	beta-gamma	-			
" 7	SI	beta-gamma	-			
" 8	SI	alpha	-			
" 11	ND	ND	ND	HS		
" 12	SII	alpha	-			
" 13	SII	beta	-			
" 15	SII	beta	-			
" 17	SI	gamma	+			
" 20	ND	ND	ND	HS		
" 22	ND	ND	ND			+
" 24	SII	beta	ND	L		+
" 25	ND	ND	ND			+

SI immuno chemically gamma-2 globulins
SII " " a beta immunoglobulin ? equivalent Beta 2 A in man

Pw anti serum
HS horse serum
L limulus

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Myeloma globulins are related antigenically to SI or SII, or very rarely both. The normal SI and SII proteins contain different antigenic determinants. Myeloma globulins may contain different antigenic determinents, or lack antigenic determinents. Using a series of the Pw anti sera, an immuno chemical characterization of the myeloma globulins of BALB/C mice can be made. A similar degree of genetic control cannot be achieved with myeloma globulins sampled at random from heterogeneous populations.

With an inbred strain of mice, members have a nearly identical genotype and the plasma cells of that strain presumably should have an identical somatic cell genotype. The purpose of the study is to learn how individual plasma cell neoplasms acquire their stable, heritable specific protein production. It is not decided whether this results from some pathologic consequence of carcinogenesis or whether the tumors are neoplasms in clones of plasma cells.

2 b.) In collaboration with Dr. E. L. Kuff, Laboratory of Biochemistry, NCI

An immuno chemical analysis of the microsomes of plasma cell neoplasms (presumed site of protein synthesis in these cells) is underway.

It is hoped these techniques will be of use in showing the differences between RNP particles. RNP particles can be caused to migrate in agar-gel electrophoresis. Studies of the proteins attached to the microsomes as well as antigenic composition of the microsomes are in progress.

The myeloma globulins of three of the neoplasms precipitate when whole serum is diluted in low ionic strength media. This has provided a simple means for the purification of the myeloma globulins. Rabbit precipitins are being prepared against these proteins. This antibody is to be used also in the characterization of the antigenic structure of the microsomes.

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2 c.) Studies on the antibody activity of myeloma globulins

This part of the project requires much developmental work of a technical nature, particularly in the area of establishing the type or types of antigen-antibody systems to be employed. The project is of considerable importance, however, to the concept of the myeloma globulin.

Plasma cell neoplasms which have occurred in mice injected with IS, and also given immunizations, have been transplanted. The serum from these mice is being studied for the presence of precipitins to the original antigen employed.

At this time no antibody producing neoplasm has been found but the work done in this direction, though considerable has had only a minimal yield of possible antibody producing tumors.

Certain natural antibodies of mice are known. Dr. M. Landy is examining various sera for this antibody activity.

3. Studies on methylcholanthrene (MCA) induced leukemia of strain $DBA^f/_2$ and BALB/C mice

Approximately 80% of one-month-old $DBA^f/_2$ mice painted with MCA (20 paintings given over a 7-week period) develop generalized leukemia. No leukemias appear in strain BALB/C mice which have been painted in a similar way. The F_1 hybrids of BALB/C and $DBA/_2$ also have not developed leukemia, after skin painting. The first generation backcross to $DBA^f/_2$, ($DBA^f/_2$ x (BALBx$DBA^f/_2$) F_1), have an incidence of approximately 25% leukemia. These studies are being extended to a family study of 2nd generation backcross mice.

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Part B.

Honors, Awards and Publications

Potter, M., and Fahey, J. L. Studies on Eight Transplantable Plasma-Cell Neoplasms of mice. J. Nat. Cancer Inst., 24: 1153-1164, 1960.

Potter, M., and Robertson, C. L. Development of Plasma-Cell Neoplasms in BALB/C Mice after Intraperitoneal Injection of Paraffin-oil Adjuvant Heat-killed Staphylococcus Mixtures. J. Nat. Cancer Inst., 25: 847-858, 1960.

Fahey, J. L., Potter, M., and Nathans, D. Myeloma Proteins and Macroglobulins Associated with Plasma Cell Tumors in Experimental Animals. Acta Union Internat. Contr. Cancrum, XVI: 1137-1140, 1960.

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Serial No. 427 NCI (b)
(1) Laboratory of Biology
(2) Leukemia-Studies Section
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Part A.

Studies on biochemical mechanisms of sensitivity and resistance to antimetabolites

Elizabeth P. Anderson

Man years (calendar year 1960)
Total: 2
Professional: 1
Other: 1

A. Studies on azaserine

This is a continuation of work reported in previous years on biochemical differences in the metabolism of azaserine-sensitive and -resistant lines of the plasma cell neoplasm 70429.

1. The azaserine-resistant Az-R-1a line, which we have previously studied biochemically, has been compared with the sensitive line with regard to capacity for concentrative cell transport and intracellular breakdown of azaserine. The basic aspects of this study were completed in February-April of this year, at Sloan-Kettering Institute (Rye, N.Y.), in collaboration with Dr. John Jacquez; Dr. Jacquez has previously studied amino acid transport, and specifically azaserine transport, in certain other types of cells. Azaserine is apparently carried into cells by a typical amino acid transport system, so that this constitutes, in fact, a true metabolic mechanism, involving the capacity to actively concentrate the compound within the cell. Previous studies in our laboratory had provided evidence for an "intact cell factor" in the resistance mechanism in this cell line, in that the difference between sensitive and resistant lines, in degree of azaserine action, was greated in intact cells that it was in cell-free extracts. It was, however,

Part A.

necessary to study specifically the entry of the compound into the cells to determine if this "factor" could be attributed to greater impermeability of the resistant cells to the drug; there is an unfortunate tendency to assume, without much evidence or justification, that all "intact cell" effects are due to differences in permeability of the cell membrane.

In the results attained, no major differences between the two lines were observed in capacity to concentrate azaserine within the cell; certainly there was no impermeability to azaserine in the resistant cells. Quantitative differences were found; the sensitive line appeared to have a slightly higher initial velocity of uptake, whereas, over a longer period, the resistant cells surpassed the sensitive ones in the final intracellular concentration which they could maintain. Both of these differences were very small in magnitude, and it was concluded that differences in transport capacity could not account for the level of resistance to the drug observed in the 70429-Az-R-1a line. The experiments also determined the extent of intracellular breakdown of azaserine in the two cell types, and here too no critical differences were found; it was therefore concluded that increased breakdown of the drug by the resistant cells could likewise not account for the resistance observed. The studies have subsequently been extended in Bethesda, with some check on reproducibility in methodology between the two laboratories and in the tumor material at different times. Additional studies have also been begun on reversal of azaserine transport by normal amino acids in the two lines, since remaining explanations for an "intact cell effect" include differences in pool size of these normal metabolites or in their capacity for reversal of antimetabolite transport. Some differences in pool size of nucleotides have also been indicated by the data accumulated and will also be studied.

2. Two new azaserine-resistant sublines of 70429 have been developed and partially characterized biologically; the level of resistance in both of these lines is high. As in the case of the Az-R-1 line, previously isolated by Dr. Potter of this

Part A.

section, each of these resistant lines also arose in a single transfer generation, indicating development of resistance in a "single step".

B. Studies on fluorouracil

1. This work is being carried out in collaboration with Dr. Law of this section, and Dr. Wallace Brockman of Southern Research Institute. The development and biological characterization of several fluorouracil- and fluorouridine-resistant lines of the mast cell neoplasm P815 has been carried out in Dr. Law's laboratory, Dr. Brockman has been doing tracer studies to determine the patterns of incorporation of pyrimidine nucleotide and nucleic acid precursors in these lines, and our laboratory is carrying out enzymatic studies on metabolic mechanisms in correlation with the biological and isotope studies. Our first aim has been to explore the biosynthetic capacities of fluorouracil-sensitive and -resistant lines to detect any decreased ability of the resistant line to synthesize pyrimidine nucleotide derivatives (a pattern which has been found for resistance to various other purine and pyrimidine analogs). Uracil anabolism is being studied in both lines, using specific assays for uridine phosphorylase and uridine kinase; these two enzymes probably constitute the major pathway for the biosynthesis of uridylic acid in mammalian tissues. There is good evidence that fluorouracil is anabolized to fluorouridylic acid by the same enzymes, and fluorouracil will also be specifically studied in the same system. Uracil anabolism by an alternative pathway involving reaction with 5-phosphoribose-1-pyrophosphate was also investigated in our preparations and found to be negligible; this activity was compared with those of the purine nucleotide pyrophosphorylases, which are very active in these tissues. Considerable effort has been spent developing assay methods for these various enzymes and improving their sensitivity, and new information has been accumulated about details of these enzymatic reactions. Our results to date show that cell-free preparations of the resistant line possess both uridine phosphorylase and uridine kinase activities; this has also been indicated

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by the isotope incorporation experiments. However, preliminary data from our enzyme studies indicate that both enzymatic activities, but especially uridine kinase, may be weaker in preparations from the resistant line than in comparable ones from the sensitive line. It is planned to do much more in the way of quantitating the assays to define these differences more exactly and to explore the various possible explanations for them.

C. Studies on the metabolism of ribonucleic acid

1. Publication was made of our studies on a phosphodiesterase purified from ascitic leukemia L4946 (see Part B). This enzyme could be detected in all tumor tissues examined, apparently because these tissues differed from non-tumor tissues in being remarkably free of enzymes for mononucleotide catabolism. As discussed in last year's report, the enzyme represents a new type of phosphodiesterase, with an unusual type of cleavage and a unique substrate specificity. It therefore promises to become a new tool for research on oligonucleotide and nucleic acid structure and, to this end, has attracted considerable interest from other laboratories.

D. Biochemistry of cancer

1. The beginning months of this year were spent completing the chapter on Biochemistry of Cancer for the 1960 Annual Review of Biochemistry, which was published in July (see Part B). This review covered recent work on carcinogenesis (with emphasis on genetic, immunological, chemical, and viral aspects), on tumor viruses, and on antimetabolites active as anticancer agents; in the latter area attention was concentrated on mechanisms of action of these agents and mechanisms of resistance to them. The aim of the review was to present extensions of concept as well as critical evaluation of selected literature in these areas.

Part B Included Yes X No

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Part B.

Honors, Awards and Publications

Anderson, E. P., and Law, L. W., Biochemistry of Cancer. Annual Review of Biochemistry, 29: 577 (1960).

Anderson, E. P., and Heppel, L. A., Purification and Properties of a Leukemic Cell Phosphodiesterase. Biochim. et Biophys. Acta, 43: 79 (1960).

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1. (Laboratory of Biology)
2. (Leukemia-Studies Section)
3. (Bethesda 14, Md.)

Part A.

Factors effecting transplantation of hematopoietic tissue in irradiated mice with particular reference to alteration of experimental lymphomas

Delta E. Uphoff

Man Years (calendar year 1960)
Total:2
Professional: 1
Other: 1

In past reports emphasis was placed upon the genetic factors influencing protection against lethal total-body irradiation by a post irradiation inoculation of bone marrow. More recently this work has been extended to include the mid-lethal dosage level. Sufficient data is now available so that generalizations and comparisons can be made as to the effects of the dose of irradiation on the successful transplantation of bone marrow at the mid-lethal and lethal dosage levels. The post irradiation inoculation of genetically compatible marrow (marrow donor and host share the same histocompatibility -2 (H-2) phenotype) will result in protection of the irradiated mice with no apparent immunological response at either dosage level. The available information indicates that these protected animals are chimeras consisting of an irradiated host of one genotype and a hematopoietic system of the donor genotype. The inoculation of genetically incompatible marrow (marrow donor and host of different H-2 phenotypes or of the $H\text{-}2^k$ phenotype), gives somewhat different results. Following lethal irradiation incompatible marrow may give rise to a graft versus host reaction ("secondary disease") which may result in death of the irradiated marrow inoculated mice after apparent recovery from the acute irradiation damage. The severity of the reaction and degree of mortality varies with the strain

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combinations and is probably a function of the antigenic incompatibility of the strains employed. The inoculation of genetically incompatible marrow following exposure to a mid-lethal dose of irradiation may result in what appears to be a host versus graft reaction which may result in an early death of the treated mice. Although the exact cause of death has not been determined, it is clearly a function of the phenotype of the irradiated host. It occurs following the irradiation and marrow inoculation of two different strains of the H-2^k phenotype (other strains of the H-2^k group have not been tested). This lethality of the treatment has not been observed in irradiated hosts of the H-2^a, H-2^b or H-2^d phenotypes.

The acquisition of technical assistance during the latter quarter of 1960 will make possible the continuation of this study as well as the initiation of new experimental procedures such as hematologic and serologic techniques which may lead to a better insight into factors which will influence the survival of the treated mice.

A. Adult marrow transplantation

1. It has been established that in marrow transplantation following lethal irradiation between strain combinations of the H-2^b or H-2^d groups there is a lack of strain specificity but a dependence upon H-2 specificity. The H-2^k group of which five inbred strains are available is more complex. There are varying degrees of reaction resulting in decreased survival when host and donor are strains of the H-2^k group. Attention was focused upon an attempt to determine the degrees of antigenicity between strains of this histocompatibility-2 group. Two of the strains of this group are genetically high leukemic strains so that this study is an integral part of a project reported elsewhere (D1). Inbred strains as well as F_1 hybrids between different strains of the H-2^k group are being studied. The observation that marrow transplantation between two different hybrids of strains of the H-2^k group (a combination involving four different parental strains) gave better long term

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survival than was observed between these same inbred strains or between the parental strains and the hybrids, led to an extention of this project to include two new aspects namely, that of a gene dosage effect and the existence (or absence) of an F_1 hybrid substance .

Van Bekkum, et al. (J. Cell. & Comp. Physiol, 50: Supp. 1, 139, 1957) observed the absence of a reaction in an F_1 hybrid treated with parental strain marrow although their parental strains were clearly incompatible with each other. To explain these results in the light of other genetic data, a gene dosage phenomenon was postulated (Uphoff and Law, JNCI, 22: 229, 1959). No other evidence for a gene dosage effect existed until the aforementioned observation on results between hybrids of the $H\text{-}2^k$ group. A survey of available F_1 hybrids is underway to determine to what extent a gene dosage effect may influence the successful transplantation of marrow in the lethally irradiated mouse. A corollary to these experiments is the establishment of evidence for the existance (or absence) of an F_1 hybrid substance. Owen (J. Med. Educ. 34: 366, 1959), postulated the possible existence of an antigenic substance which might be present in the F_1 hybrid but absent in either parental strain and which might influence marrow transplantation. Although there is no good evidence available that would support such a hypothesis, it seemed timely to undertake this aspect of the work in conjunction with a study of the effect of gene dosage . Although more data is necessary before any conclusions can be drawn, of the several F_1 hybrids tested, the only evidence for a gene dosage effect occured in the hybrid combinations of $H\text{-}2^k$ and there has been no evidence indicating the existence of an F_1 hybrid substance.

2. For the study of the genetic factors influencing protection by marrow inoculation following mid-lethal irradiation a dose of 500 r or 550 r total body X-irradiation was used. This dosage would be expected to be lethal to from 10% to 30% of the mice within 30 days post irradiation. The post irradiation inoculation of homologous marrow protected

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95 to 100% of the treated mice if the marrow donor and irradiated host were of the same H-2 phenotype in eleven different strain combinations. In twenty-three strain combinations in which the irradiated host was of an H-2^a, H-2^b or H-2^d phenotype, no early mortality was observed. In twenty of these combinations there was a significant amount of long term protection afforded by the marrow inoculation. In two irradiated hosts of the H-2^k group (CBA and C3H) treated with marrow of an H-2^b or H-2^d phenotype, a marked early lethal effect was observed. When homologous marrow from H-2^k or H-2^a mice was used no lethal effect was observed. In the past (Trentin, P.S.E.B.M. 93: 1956, and Van Bekkum, J. Cell. Comp. Phys. Supp: 1, 139, 1957) this lethal effect was attributed to a host versus graft reaction. It is not clear why CBA mice treated with Strain A marrow (H-2^a) did not exhibit this early lethality. In this case, 96% of the mice survived 14 days but later developed a syndrome resembling secondary disease resulting in a greatly reduced long term survival. More mice in this group survived 90 days than in combinations of H-2^b or H-2^d marrow in H-2^k irradiated hosts. The full significance of this observation is not immediately apparent. It may be concluded, however, that in most genetic combinations the inoculation of homologous marrow following mid-lethal irradiation protected the irradiated mice from the lethal effects of the irradiation and this protection is long lasting, although chimeras were established only between strains of the same H-2 phenotypes. In several strain combinations a syndrome developed which closely resembled "secondary disease". This late occurring syndrome and the cause of the early mortality in the two strains of the H-2^k group will be the subject for further investigation. Preliminary experiments indicate that strains AKR, C58 and ST/JSn may not exhibit the marked lethal effect seen with CBA and C3H mice. The chimeric state of these treated mice is being tested and will be discussed elsewhere (D3) along with a consideration of the significance of these observations.

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B. Preclusion of the reaction

1. The preclusion of the reaction following treatment with A-methopterin (Uphoff P.S.E.B.M. 99: 651, 1958) gave hope that this might be a genetically non-specific method which might have practical clinical application. The marked sensitivity of the irradiated mice to the toxic effect of antimetabolites led to a new approach to the problem namely that of attempting to produce "tolerant" donors or hosts by a pretreatment method. In the lethally irradiated mice inoculated with homologous marrow and treated with A-methopterin a permanent state of immunologic non-reactivity is achieved since these surviving mice have been demonstrated to be chimeras. Starting with the premise that treatment with A-methopterin in the presence of a specific antigen may lead to a "tolerant" state in the adult mouse, attempts were made to produce "tolerance" by treating mice with A-methopterin and a strain specific antigen in the form of a crude tissue brei of spleen and thymus. Tests for specific non-reactivity were done by transplantation of a neoplasm of the same strain as the antigen. Although this test system is not ideal, it proved a quick and efficient method of screening a large number of mice on different treatment schedules in an attempt to arrive at the optimum experimental procedure that will effectively produce a state of specific non-reactivity without the complicating factors reported previously (1959) when the "tolerant" state was tested by marrow transplantation in the irradiated host. By this method a system was set up which allowed for the growth of a mammary gland tumor in 100% of the treated mice. In this case the host and donor were of the same H-2 phenotype. In the same genetic systems one group of pretreated mice tested nine months after treatment with what is now considered a less than optimum drug-antigen treatment, 5 out of 15 mice were "tolerant" and died of progressive tumor growth. Mice treated with antigen only were resistant to tumor growth, while with drug treatment alone, 1 out of 14 had positive tumor test. Recently it has been possible to grow an $H\text{-}2^k$ reticulum cell sarcoma in 5 out of 13 $H\text{-}2^d$ mice pretreated with A-methopterin and $H\text{-}2^k$ tissue antigen.

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It is not known how many strains and transplantable neoplasms will lend themselves to this type of manipulation. For the genetic system which has been employed in this study the following factors have proved critical, the amount of antigen and drug dosage employed as well as the time relationship between the administration of the drug and antigen. Reduction in the drug dosage or the accumulated amount of drug or antigen reduces the number of positive tumor tests. It also appears that the greater the genetic difference between host and donor the more toxic the drug-antigen treatment. Upon subsequent transplantation the neoplasms which grew out of strain occasionally grew in the foreign strain but the irregularity with which this occurred was attributed to a possible alteration in the antigenicity of the tumor rather than an enhancing factor. Although these are preliminary results and this approach will be studied more thoroughly in mice, the urgent clinical need for a method other than irradiation for the preparation of the host for a homotransplant made it seem desirable to attempt this procedure in dogs. A collaborative project has been set up with Drs. J. W. Ferrebee and E. D. Thomas of the Mary Imogene Bassett Hospital in Cooperstown, New York, in which littermate dogs are being pretreated by a method similar to that used in mice for kidney transplantation. This group has had previous experience in treating two dogs which developed "secondary disease" following lethal irradiation and marrow transplantation with A-methopterin in a manner similar to that used in mice. When a sufficiently predictable system is developed in which it is possible to cross the H-2 barrier these pretreated mice will be used as marrow donors for lethally irradiated hosts to test the effect of pretreatment upon the course of secondary disease.

C. Transfer of immunity by marrow graft

1. In collaboration with Dr. Victor Haas (NIAID-84) experiments attempting to transfer immunity to lymphocytic coriomeningitis (LCM) virus by means of lethal total body X-irradiation and marrow inoculation were completed. In these experiments LCM-immune, nonimmune, and LCM-carrier mice were

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exposed to a lethal dose of irradiation and protected by marrow inoculation from immune or non-immune donors. Subsequent challenge of the surviving chimeras with potent homologous virus established that under the conditions of the experiment immunity was a function of the host and not the transplanted tissue. These results are at variance with other types of experimental data. The following hypothesis was proposed as a possible way of reconciling the divergent concepts of host-graft interaction: 1) antigen introduced before irradiation established within the host a pattern of immunological response which is usually, though not invariably radiation-resistant, and 2) the irradiation may fix the immune mechanism of the host to the condition existing at the time of exposure. After irradiation, the host tissue responds with immunologic competence only to those antigens with which it had pre-irradiation experience. 3) The host does not respond immunologically to new antigenic stimuli or to stimuli to which it would not normally respond. 4) Under conditions in which immunological competence of the marrow graft exists, the graft may react against the foreign antigens of the irradiated host producing "secondary disease", 5) such marrow may also impose on the host its own immunologic mechanism.

2. In a previous report (Uphoff and Law, J.N.C.I., 22: 229, 1959), a threshold effect was postulated in conjunction with a gene dosage effect to explain the absence of a graft versus host reaction under genetic conditions which otherwise would have been expected to produce an immunological response. In an attempt to demonstrate the existence of a threshold effect in this immune response, donor and hosts sharing the same H-2 phenotype (either $H\text{-}2^b$ or $H\text{-}2^d$) were selected. In these combinations no grossly visible immune response is observed following marrow transplantation in the lethally irradiated host (A1). Marrow donors were immunized against the host strain by pre-treatment with a crude tissue brei of spleen and thymic cells. This immune marrow protected the irradiated host from the acute irradiation damage but a typical "secondary disease" syndrome followed the initial recovery. This observation has been made on several different genetic combinations. Body-weight

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curves were made on one experimental group. The curves are identical with those for a typical "secondary disease" when host and donor are of different H-2 phenotypes. Preimmunication against histocompatibility genes other than H-2 results in a visible, lethal reaction thus demonstrating the existence of a threshold effect for this immune response. This experiment clearly demonstrates the role of the marrow graft in this immune response. Experiments are in progress using pre-immunized irradiated hosts in the same genetic combinations. Preliminary results indicate that an early death may result from this treatment. This would suggest that in the preimmunized host the irradiation does not alter the immunity and the marrow graft is rejected before it can protect the irradiated host from the acute irradiation damage. Similar experiments are also in progress testing the effect of preimmunization of host and/or donor in mice exposed to a midlethal dose of irradiation in an attempt to establish the type of immune response responsible for death after marrow inoculation at this dosage level, i.e., a host versus graft reaction, a graft versus host reaction or a combination of both reactions.

D. Chimeras

1. The previously reported study of the histocompatibility patterns of the malignant lymphomas arising in the irradiation-induced chimeras was continued. Since the experiments attempting to alter the incidence of leukemia in the genetically high leukemic AKR strains were extended to include marrow transplantation from the four available H-2^k strains in AKR mice ranging from three to twelve months of age, most of the tests involved the AKR strain in one capacity or another. For purposes of brevity the two projects have been consolidated into a single report. The neoplasms in the lethally irradiated, marrow inoculated mice are primarily of donor tissue origin (124 positive tumors of donor genotype to date). The latent period for the induction of these lymphomas was 2.5 to 11 months post-irradiation. Since leukemic mice as well as mice

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in a preleukemic stage were used in these experiments a large number of the tumors of host phenotype represented cases in which the leukemic cells were not destroyed by the exposure to 700 r X-irradiation. Of the 46 neoplasms of host phenotype 36 tests represent cases of this type. The latent period for the regrowth of leukemic cells after irradiation was 1 to 4 months. The 10 remaining cases are considered to represent reversal to host genotype after initial protection by the donor marrow. The latent period for the regression of the marrow graft, reversal to host genotype and the development of leukemia was 3 to 7 months post-irradiation. There was an overlapping period during the third and fourth months when the differentiation between these two processes was arbitrarily made on the basis of the age of the mice at the time of treatment. Although there may be errors in subdividing the host type neoplasms, they are fewer in number than those of donor genotype indicating that although reversal to host phenotype may occur it is not common for a well established marrow graft to regress. An observation of greater significance is the relatively large number of neoplasms of donor tissue origin. A few AKR mice treated as late as 11 months of age developed neoplasms of donor tissue origin, 3.5 to 4.5 months after treatment. More mice of this age range are currently being tested. Other experimental groups still in progress are thymectomized mice protected from lethal irradiation by marrow inoculation from the Street Strain. Preliminary data indicates that they do not develop lymphomas. The AKR-ST chimeras develop primarily thymic neoplasms while AKR-CBA chimeras may develop lymphomas of nonthymic origin. Thymectomized AKR mice will be protected with CBA marrow to study the effect of thymectomy and marrow transplantation on the non-thymic neoplasms observed in this genetic combination. Other irradiated H-2^k mice (either C3H, CBA, ST or C58) inoculated with AKR or F_1 hybrid marrow infrequently develop lymphomas. The few that have developed were of donor tissue origin. Speculation as to the significance of these experiments will not be made at this time.

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2. Mice exposed to a mid-lethal dose of irradiation and inoculated with homologous marrow were kept for 90 days so that a comparison could be made between the long-term survival at the lethal and mid-lethal dosage levels. All surviving mice were to be tested for their chimeric state by transplantation of neoplasms or skin of donor strain origin into the treated mice. Progressive tumor growth or permanent skin transplantation would indicate a chimera while regression of the neoplasm would indicate a sensitization of the host to donor tissue and rejection of the marrow graft as well as the tumor graft. Several tests were completed and preliminary results indicated that when host and donor were of the same H-2 phenotype the neoplasms grew progressively and killed the host. The same neoplasm did not grow in the irradiated control mice from the same experimental group. This indicated that the neoplasm did not grow out of strain and the inhibitory effect of the irradiation upon the immune mechanism did not extend into the test period of approximately 100 days post-irradiation. In combinations in which host and donor were of different H-2 phenotypes, donor type neoplasms were rejected. Although several experiments were completed this should be considered a preliminary report since this project was temporarily abandoned when it was observed that lymphomas developed in some genetic combinations but not in others. Subsequently a study similar to that for the lethally irradiated mice was made of the incidence and histocompatibility pattern of malignant lymphomas in the different genetic combinations. Since malignant lymphomas have not been observed in combinations in which both host and donor are of the same H-2 phenotype several experimental groups of different genetic combinations will be set up and tested by skin or tumor transplantation to test the hypothesis that these mice are chimeras and do not develop neoplasms unless the donor strain is genetically high leukemic.

3. The histocompatibility pattern of the malignant lymphomas arising in mice exposed to a mid-lethal dose of irradiation and homologous marrow inoculation is quite different. Forty tests have been completed, 33 of which are of host

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tissue origin and 7 of donor tissue origin. Of those 7 neoplasms, 6 represented genetic combinations in which both donor and host were of the H-2^k phenotype, only 1 represented successful marrow transplantation across the H-2 barrier (an H-2^b neoplasm arising in an H-2^k irradiated host). At this dosage level lymphomas arise primarily in genetic combinations in which the host and donor are of different H-2 phenotypes. In H-2^b of H-2^d combinations in which and donor are of the same H-2 phenotype, lymphomas have not been observed. It would appear that although the marrow graft is capable of protecting the host against the acute irradiation damage, it does not appear to protect the irradiated mouse against this late radiation effect possible because of the regression of the graft as indicated by the lymphomas of host tissue origin. The demonstration of a "tiding over" effect of genetically incompatible marrow at this dosage level may be an experimental counterpart to theclinical observations on the marrow inoculation in the Yugoslavian accident victims in which there appeared to be clinical improvment but subsequent disappearance of donor type cells from the circulating blood indicating a regression of the "graft".

E. Multiple polyposis of the colon, a genetic trait

1. An increased amount of space was devoted to the development of the C57BL/10D line carrying the genetic trait for multiple polyposis of the colon. A large number of mice from this line are used in experiments at the lethal and mid-lethal levels. Although polyps are occasionally found in these experimental mice, malignancies have not been observed. More data is necessary both from the breeding colony and the experimental animals before generalizations can be made.

F. Etiology and pathogenesis of experimental lymphomas

1. Experimenta previously submitted as a separate report (Uphoff and Law, 1959) have now been completed. Plans are to process these data for publication within the coming year.

Part B Included Yes X No

Serial No. NCI 427 (c)
1. (Laboratory of Biology)
2. (Leukemia-Studies Section)
3. (Bethesda 14, Md.)

Part B.

Honors, Awards and Publications

Uphoff, D. E., Haas, V. H. Immunologic response to lymphocytic choriomeningitis virus in lethally irradiated mice treated with bone marrow. J. Nat. Cancer Inst., 25: 779-786, 1960.

Serial No. 428 (a) -Project 1
1.Laboratory of Biology,
2.Tissue Culture Section,
3.Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Cinemicrographic Studies of Tissue Cells in vitro.

Principal Investigators: W. T. McQuilkin, and W.R. Earle.

Other Investigators: V. J. Evans, K. K. Sanford,

Cooperating Units: None.

Man Years (Calendar Year 1960)
Total:
Professional:
Other:

Patient Days (Calendar year 1960)

Project Description: Objectives: 1) A study of the alteration of morphologic and behavioral characteristics of strain NCTC 929 (strain L) cells resulting from long continued growth in chemically defined, protein-free medium, NCTC-109 in vitro. This study has been expanded to investigate the effect on the altered cells of returning them to the original protein medium for an extended period. 2) To determine feasible, qualitative and quantitative techniques of cinemicrographic film analysis that can be applied generally to the study of other cell strains. For this purpose, the present investigation is serving as a pilot study of film-analysis procedure.

Part B included Yes x No

Methods Employed: Detailed study of numerous films on a projecting machine and study of enlarged transparencies of selected film frames are made. Qualitative observations and comparisons are made. Where enumeration, timing, tracing, and measuring of cellular events are possible, quantitative data are obtained and analyzed statistically. Cell characteristics being analyzed quantitatively include intermitotic intervals, cytokinetic periods, growth rates, and migration rates. An attempt is also made to correlate findings by film analysis with observations made on living cultures.

Major Findings: Standardized and feasible techniques for accurately characterizing and comparing intermitotic intervals and culture growth rates of cell strains have not yet been fully worked out. Perhaps the major contribution of the cinemicrographic approach to cytology will be to emphasize the extreme variability of behavior among cells of pure line populations and to point up the dangers of too broad generalizations of cell behavior.

In the extension of the study to include the cell strain returned to the original medium the findings indicate a marked tendency of most cell characteristics studied to revert to those of the original strain. These results appear to indicate that whatever the phenomena of adaptation to chemically-defined medium might be, the observed changes are not necessarily permanent but are often of a reversible nature.

Significance: The potentialities, limitations, and film analysis techniques of cytological population studies by cinemicrography are being better defined. Some basic data on cell behavior of three related strains have been assembled and are being analyzed and compared. Therefore, this study is serving as a prototype to set up guide lines and criteria for future studies in which cinemicrographic comparisons will be made between other cell strains.

Project Description: Cinemicrographic comparison of the growth and transformation of C3H mouse embryonic cells from non-malignant to malignant cells. This work is related to project 428(c) reported elsewhere.

Objective: To determine whether cell and culture characteristics analyzable by moving pictures can be correlated with histological and cytological evidence of the onset of malignancy of *in vitro* cells as determined by eye implants in the mouse strain of origin.

Methods Employed: Simultaneous films of *in vitro* cultures were made at weekly intervals. Cells were drawn from the same cell suspensions from which eye implants were made. About 3,000 feet of film were taken but have not as yet been analyzed in detail by methods discussed above.

Serial No. 428 (a)
Project 1

Proposed Course of Project: To complete present investigation of description and comparison of cell strains maintained in different media but of common single cell origin. To more nearly standardize and determine feasible and significant methods of film analysis. To more nearly standardize culture and photographic conditions as appears necessary in light of present film analysis experience. To continue with such comparative studies as 1) the malignancy transformation study mentioned, and 2) the effects of virus on in vitro cultures.

Part B Included Yes X No

Part B Honors, Awards and Publications:

A preliminary report entitled "Cinemicrographic comparison of NCTC clone 929 (strain L) mouse cells grown in protein medium and in chemically defined protein free medium"was presented at the Annual Tissue Culture Association Meeting in Chicago, April 6, 1960. Two films were shown to accompany this talk.

Serial No. 428(b) -Project 1
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 1, Title: Rapidly agitated fluid suspension cultures for the growth of mammalian cells.

Principal Investigators: J. C. Bryant, E. L. Schilling, and W.R.Earle.

Other Investigators: H. Montes de Oca.

Cooperating Units: None.

Man Years (calendar year 1960)
Total:
Professional:
Other:

Patient Days(calendar year 1960)

Project Description: This continuing project is the study and improvement of devices and methods for growing mammalian cells, particularly established strains of monkey kidney epithelium clone strain NCTC 3526, in rapidly agitated fluid suspension culture, in protein-free chemically defined media.

Objectives: The general objective is to define and control more closely factors influencing growth and maintenance of this strain of cells under such defined conditions. Emphasis this year has been placed on 1) transfer to the new constant temperature room (324a) of the entire shaker assembly, gas metering device and gas tank, so maintaining all at a uniform temperature; 2) study and use of smaller culture flasks than had been used previously, in order to allow handling of larger numbers of cultures and direct centrifugation of the cultures at fluid change.

Part B included Yes __ No x

Methods Employed: The special 1½-liter shaker flasks, adapted for continuous gas phase renewal, and the solenoid-valve gas-metering device have been described in earlier reports. Smaller shaker flasks, 500-ml.and 250-ml.in size, first used by Bakken, Evans et al of this laboratory (Am. J. Hyg., in press) in late 1959, can be centrifuged directly at each fluid change without the necessity for pipetting the cells into centrifuge tubes and back into the culture flask. This greatly facilitates technical procedures. Continuous aeration is accomplished through two cotton filter tubes inserted through the single stopper.

Major Findings: 1) Installation of the entire assembly in the constant temperature rooms did not cause significant changes in the thermostatic control of temperature of the room, i.e., the contribution of heat by the electric motor was compensated by minor changes in temperature controls. 2) Loss of water from each culture by evaporation has been greatly reduced by the uniformity of the temperature (37.5°C) at which all components of the assembly are maintained. 3) Smaller culture flasks (125 ml.) of the same shape (flat-bottom boiling flask) but smaller size than has been used in previous work with the shaker were demonstrated to be satisfactory for allowing rapid proliferation of the monkey kidney epithelial cells under study, whereas these cells had never shown significant proliferation under the fluid suspension conditions previously studied.

Significance: 1) A major difficulty with large agitated fluid suspension cultures is the lack of significant replication. Further, limitations of time, labor, media, and space do not allow more than 5 or 6 such large cultures (400 ml. maximum fluid volume) in one experiment. Only gross differences can be detected between cultures in flasks of 1½-liter size in experiments lacking adequate replication. 2) Small flasks of 125-ml. size, such as those used in recent experiments, can be handled efficiently in replicate culture experiments at 24 to 36 cultures of 30-35 ml. fluid suspension volume. 3) With these small rapidly agitated cultures it has been possible, for the first time to maintain clone strain NCTC 3526, monkey kidney epithelial cells, in rapid cell proliferation, on protein-free, chemically defined medium. 4) These results open up new avenues of exploration in studies of cells in agitated fluid suspension cultures.

Proposed Course of Project: 1) Improvement of the procedures and device is a continuing objective. 2) A major long-term objective is to determine more accurately the conditions necessary for maintaining a rapid and efficient harvesting-subculturing cycle,for various cell strains and in various culture media, in addition to those for which such a cycle has been obtained.

Part B Honors, Awards, and Publications:

Publications other than abstracts:

The two papers listed under Project 428 (b-2) incorporate, to some extent, results from this project.

Honors and awards:

1) Invitation to W. R. Earle to organize a Symposium on Mass Cell Culture, Division of Agriculture and Food Chemistry, Fermentation Subdivision, American Chemical Society, Fall Meeting, September, 1960; this Symposium was postponed to September, 1961, and he has been asked to organize it for that time.

Serial No. 428 (b) - Project 2
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 2, Title: The growth potentiating effects of various substituents on chemically defined medium NCTC 109 with certain established cell strains in rapidly agitated fluid suspension cultures of various types and sizes.

Principal Investigators: J.C. Bryant, V.J. Evans, E.L. Schilling, and W. R. Earle.

Other Investigators: B.B. Westfall, E.V. Peppers and H.A. Kerr.

Cooperating Units: Robert A. Taft Sanitary Engineering Center, Water Supply and Water Polution Research, Cincinnati, Ohio, and R. E. Stevenson, Grants and Training, N.C.I.

Man Years (calendar year 1960)
Total:
Professional:
Other:

Patient Days(calendar year 1960)

Project Description: This continuing project is a study of the growth-potentiating effect on chemically defined medium NCTC 109, of Methocel or other supplements with certain cell strains in agitated fluid suspension cultures of several different types.

Objectives: The objective is to grow mammalian cells of selected established strains in agitated fluid suspension cultures, on chemically defined medium NCTC 109 without serum or protein hydrolysate supplement, by substituting either chemically defined polymers such as methylcellulose or certain small molecule additives. Emphasis was placed this year on a monkey kidney epithelial clone strain, NCTC 3526.

Part B included Yes ____ No x

Methods Employed: The type of agitated cultures and the basic procedures are considered in Project 1, Serial 428(b). In addition, the special roller tube equipment with speeds of rotation graded between 19 to 2400 rph, was used for certain experiments. This assembly is described in J. Nat. Cancer Inst. 14: 853, 1954.

Major Findings: The protective effect of methylcellulose on cells of three established mammalian cell strains (of mouse, monkey, and human origin respectively) on chemically defined medium NCTC 109 in agitated fluid suspension cultures was reported last year. This has been studied further.

Cells of mouse fibroblast strain 2071 (L) have proliferated at a steady but rather slow logarithmic rate when the walls of the flask were coated with silicone (hydrophobic). A paper on this phase of the study is to be published in January 1961.

A more detailed outline of the experimental results is summarized as follows. Cells of monkey kidney clone strain NCTC 3526 have been used in many experiments with both shaker cultures and roller tubes, in an attempt to define factors which had prevented proliferation. As stated in last year's report, these cells in agitated cultures on chemically defined medium with methylcellulose behaved in an anomalous manner. The cells remained healthy in appearance either as single cells as, cluster or clumps, but showed little if any cell proliferation over several weeks. This year, the working concept was tried, that in large 1½-liter flask cultures subjected to rotary agitation at about 8600 rph stresses occurred which were too great to allow these cells to proliferate.

A series of replicated roller tube culture experiments was carried out with silicone-coated tube cultures at various rates of rotation to find the critical "breaking speed" of the cells. Speeds of tube rotation used were in the range from 19 to 2400 rph. Even at the highest of these speeds in roller tubes stresses imposed on the cells were much less than in shaker cultures. The silicone coat appeared to be only partly effective in keeping cells from attaching to the walls: small adherent clumps or sheets of cells formed at various sites on the silicone wall, in a somewhat unpredictable manner.

The following factors were tested by replicated roller tube cultures of monkey kidney cells during 10- to 14- day experiments. 1) Rate of tube rotation; 2) Concentration of Methocel, 50 cps viscosity grade; 0.12% and 0.41%; 3) Spermine as supplement instead of Methocel; 4) Folic acid in high concentration as supplement instead of Methocel. The effect of tube rotation rate was fairly clear cut in several experiments with monkey kidney cells. Of the seven speeds used, 1200 and 600 rph gave somewhat higher proliferation rates than did speeds of either 2400 rph or 300

rph or less. The highest speed was more effective than the lower speeds.

Two concentrations of Methocel of 50 cps viscosity grade gave higher proliferation rates of monkey kidney cells than did either medium NCTC 109 alone or medium 109 supplemented with either spermine or folic acid. The higher concentration, 0.41%, gave slightly higher proliferation rates than did the 0.12% concentration which had been used for experiments with cell strain 2071 last year.

Spermine has been found by Mager (Nature 103: 1827, 1959) and others to influence the permeability of the cell walls of certain bacteria by preventing osmotic lysis, when used at concentrations from 0.2 to 1.0 micromole per ml. This finding suggested a trial of spermine for its effect on the walls of monkey kidney cells in roller tube cultures. In several experiments, spermine at concentrations within the range used by Mager showed no well-defined protective effect and was inhibitory in some cases.

Folic acid supplementation of medium NCTC 109 was suggested by the finding of Lieberman, Youngner, and Ove (Science 131: 936, 1960) that monkey kidney cells in static culture have a relative high folic acid requirement (about 2×10^{-3} micromole per ml.) for growth, about 35 times the concentration in medium NCTC 109. Folic acid at two high concentrations, 20x and 40x, gave no significant and consistent increase in cell proliferation of monkey kidney cells in roller tubes.

Shaker flask studies with monkey kidney cells, such as those reported last year, were continued, using Methocel supplement with medium NCTC 109. Most experiments were carried out with 2 to 4 siliconed 500 ml flasks (which were centrifuged directly) instead of the $1\frac{1}{2}$-liter flasks previously used. Various factors or agents which were studied included the effect of used medium from static T-60 cultures of actively proliferating cells of the same strain, and the effect of higher concentrations and viscosity grades of Methocel. Used medium was supplemented with high-glucose fresh medium before it was placed on the shaking cultures, but no cell proliferation was induced. The use of higher concentrations and grades of Methocel had similar results.

The most significant results found in the series of studies covered in both this project and project 1 (428b) are those with monkey kidney epithelium clone strain NCTC 3526 in replicated 125-ml. agitated fluid suspension cultures on protein-free, chemically defined medium. These results have been outlined in project 1: for the first time under these conditions these cells proliferated rapidly instead of merely maintaining or slightly increasing the inoculum level.

Significance: As a working hypothesis to fit together the results of these various studies and to serve as a basis for further work, it may be postulated that the degree of stress to which cells in an agitated fluid suspension culture flask are subjected is closely correlated with the size and rotation velocity of the containers used. The factors involved in this stress are quite complex and need extensive further study.

This project also is of more general significance. Since three established strains of mammalian cells have been grown at satisfactory rates for several years in static flasks on protein-free chemically defined medium, cell nutrition studies in the Tissue Culture Section (Evans project 428 (c) and Sanford 428 (e) with such media are now being extended to agitated fluid suspension cultures. Such extension is being made on the presumption that comparative studies of normal and malignant cells can be accelerated most profitably as it becomes possible to grow cell strains in protein-free, chemically defined media in agitated cultures without serum or serum hydrolysate under precisely controlled experimental conditions.

Proposed Course of Project: Studies will be continued on the supplementation of chemically defined medium NCTC 109 for the growth of other established cell strains in agitated fluid suspension cultures. Protective agents other than methycellulose will also be studied. Such potentially protective agents are carboxymethylcellulose, polyvinylpyrrolidone (PVP), and several poly-amino acids, all of which are chemically defined polymers within certain limits of molecular weight. Carboxymethylcellulose for example, in aqueous solution is a negatively charged polymer molecule which should offer opportunity to study the mechanism of binding of methylcellulose molecules to the cell walls, assuming that the divalent cations Ca^{++} and Mg^{++} act as intermediaries in this binding, as Kuchler (Exptl. Cell Research 20: 428, 1960) has suggested. Results from our studies to date indicate that the physical effects of polymers are probably more important than the nutritional effects, when such polymers are used as supplements to chemically defined media.

The recommendation in last year's report is repeated, that investigations on the use of methylcellulose as a protective agent for cells in deep-freezing methods be considered. It is suggested, at this time, however, that methycellulose be regarded as a possible supplement with glycerol rather than a placement for it. It would be unwise to disregard the possibility that use of an appropriate mixture of methylcellulose and glycerol might be better than following blindly the present use of glycerol alone for freezing cells. Infra-red spectra of certain cells and of certain media used in these studies are being determined by Mr. Bernard A. Kenner, of the Water Supply and Water Pollution Research Division, Robert A. Taft Sanitary Engineering Center, Cincinnati. This collaboration also includes Dr. Stevenson, Grants and Training, NCI, who was formerly associated with the Taft Sanitary Engineering Center. The purpose of these spectral analyses is to

determine whether or not methylcellulose is adsorbed on the cell membrane or is absorbed intracellularly by strain NCTC 2071 cells or monkey kidney cells cultured in Methocel-supplemented chemically defined medium. Interpretation of the results awaits further analyses on more carefully washed cells. Such confirmatory work is planned for an early date.

Part B Honors, Awards, and Publications:

Publications other than abstracts from this project:

Bryant, J. C., Evans, V.J., Schilling, E.L., and Earle, W.R.: "Effect of chemically defined medium NCTC 109 supplemented with Methocel and of silicone coating the flasks on strain 2071 cells in suspension cultures." J. Nat. Cancer Inst. 26(1): 000-000, 1961 (January).

Bryant, J. C., Schilling,E.L., Earle, W.R., and Evans, V. J.: "Massive fluid suspension cultures of certain mammalian cells. III. Exploratory supplementation experiments with chemically defined medium NCTC 109." J. Nat . Cancer Inst. 24(4) : 859-872, 1960.

Note: Paper 2 was mentioned in last year's report on this project, but it had not yet been published.

Serial No. 428(c)-Project 1
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 1, Title: Studies on Cell Nutrition In Vitro.

a) Cultivation of freshly isolated monkey kidney cells in chemically defined medium NCTC 109.

b) Cultivation of fresh C3H mouse fibroblasts in chemically defined medium NCTC 109.

c) Cultivation of fresh C3H mouse kidneys in chemically defined medium NCTC 109.

d) Studies on cultivation of a long term strains of epithelial cells C3H mouse liver epithelium and cervical carcinoma cells on chemically defined medium NCTC 109 by adaptation techniques and by additional chemically defined supplements.

Principal Investigators: V.J. Evans and J. C. Bryant.

Other Investigators: H.A. Kerr,
T.L.Keeler, Jr.,
G.A. Parker,
E. L. Schilling,
W.R. Earle.

Cooperating Units: None

Man Years:(Calendar Year 1960):
Total:
Professional:
Other:

Patient Days(Calendar year 1960)

Part B included Yes x No ___

Project Description: a) Cultivation of freshly isolated monkey kidney cells in protein-free chemically defined medium NCTC 109.

Objective: To determine if it is possible to grow fresh monkey kidney cells in protein-free chemically defined medium NCTC 109 so that such strains of cells can be used as a laboratory tool and model cell type for virus studies, metabolism studies and chromosome studies.

Method Employed: Monkey kidneys from which both the capsule and a reasonable amount of medulla was removed were subjected to trypsin digestion. Digestion was followed by inactivation of the enzymes by massive volumes of medium NCTC 109. No serum inactivation was employed. Fluid change schedules were varied to obtain desired populations and culture behavior.

Major Findings: The cultures cannot yet be considered as an established strain of cells. If they perform as other cell strains, they will in all probability require a prolonged adaptation period implying selection of variants or mutations or other unrecognized phenomena.

Significance: These cells if they can be established in long term cultures will be the first or at least among the first cell strains established from fresh tissue in a chemically defined medium with no large molecule supplement. They will, by the nature of the methods employed for explanting, be free from the criticism made of cells cultured on serum. Pleuropneumonia-like organism will also be excluded in this cell medium because this type of medium does not appear to propagate PPLO. Lack of serum will make the tissue extremely valuable for virus studies.

Proposed Course: This strain when established will be made available for virus studies, either collaboratively or freely to responsible investigators capable of maintaining such cells in chemically defined medium. Chromosome analysis will be made as the strain develops and will be evaluated in comparison with other cell strains.

Project Description: b) Cultivation of fresh C3H mouse fibroblasts in chemically defined medium NCTC 109.

Objective: 1) To establish new strains of C3H mouse fibroblasts in chemically defined medium so that an attempt can be made to determine whether or not cell transformations to tumor producing cells upon injection *in vivo* are influenced by the composition of the medium. 2) If the cultures continue to fail to produce tumors on injection transformations in such cells after appropriate tests should be attempted.

Methods Employed: Several series of C3H mouse fibroblasts have been set up *in vitro* in chemically defined medium. Pooled minced embryonic mouse cells have been the source with the expectation that young tissue may have the greatest potential for growth in tissue culture.

Control cultures are always established in the same medium with 10% serum added.

Results: One "strain" of cells from the pooled tissue has now been in vitro for 21 months. The cell type appears to be fibroblast-like. Waves of growth followed by waves of dying cells and then new growth is the course of events. No great increase occurs in the amount of tissue but at least there is maintenance.

Significance: Though, to date there is no real progression of growth, this survival is hopeful because a variant may develop which will adapt to the medium to grow progressively in order to give a cell system useful for the main objective.

Proposed Course: 1) To continue with strain available; 2) to develop others by technological improvement; 3) to develop improvements in medium for these cells; to pursue the obvious major objectives for the use of such a strain of cells from fresh tissue on chemically defined medium (See Serial No. 428(c), Project 2, for some results with this series and in particular the controls.)

Project Description: c) Cultivation of fresh C3H mouse kidney in chemically defined medium NCTC 109.

Objective: To cultivate fresh C3H mouse kidney epithelium in chemically defined medium of the NCTC series for carcinogenesis studies and also as test cells for development of laboratory techniques for chromosome studies. Secondarily the cells may be especially useful for virological studies.

Methods Employed: Kidney cortex cells of the C3H mouse were prepared in suspension by two methods: a) Trypsin digestion, b) Collagenase digestion. Action of both digestants was stopped by large volumes of chemically defined medium rather than usual serum inactivation. This method is used to avoid any possible effect of unknown composition of serum. Cell suspensions were planted in T-15 culture flasks and maintained on medium NCTC 109 with no addition of serum.

Results: Cell cultures in early stages of cultivation in vitro.

Significance: One of the easiest cell types to grow on chemically defined medium in this laboratory was the kidney cell of the monkey. It is hoped that the mouse kidney cell will be equally easy to grow. It offers a fairly clean cell type for perfecting techniques in chromosome studies and should have value for mouse virus studies.

Proposed Course: Continue isolation, cultivation of cells and modification of medium until problems associated with isolation of strains from fresh tissue are resolved.

Projection Description: d) Studies on cultivation of a long term strain of C3H mouse liver epithelium and Hela cells on chemically defined medium NCTC 109.

Objective: To attempt to grow 3 lines of a 12 year old strain of mouse liver epithelium and the classic Hela strain on chemically defined medium with no protein additions for characterization studies of both the liver strains in vitro, and the classic Hela strain.

Methods Employed: Depletion of serum content of medium with the hope that a hardy variant would survive has been repeatedly tried on numerous cultures of the epithelial cells grown in Carrel and T flasks. Exploratory tests were tried on some new chemicals that theoretically might be expected to stimulate growth such as lipoic acid and cholic acid; good quality oval bumin has also been used.

Results: The cells can be adapted to ready cultivation in ½% by volume of serum and ovalbumin is effective. The data on the effects of the various chemical additives are in too exploratory a stage to be anything more than suggestive.

Significance: These strains have obvious uses in the virological field. They have been characterized in many ways biochemically and morphologically. The biochemical classification to date is perhaps more exhaustive on these cell strains than on any other in tissue culture. It represents the type of biochemical data that it is possible to gather on cells cultured in vitro, and to use in endeavoring to classify them and many other cell strains. Both strains lack some usefulness because they cannot be grown in defined medium.

Proposed Course: To complete exploratory studies with the expectation that objective may be attained and significance will be clarified. An attempt will be made to learn by assessment of behavior of these cells in these additives whether or not there is a physical reason that medium NCTC 109 does not successfully maintain cells or whether it is still lacking a specific nutritional requirement for liver cells and Hela cells.

Part B Honors, Awards and Publications:

Publications other than abstracts from this subject:

Evans, V. J., Fioramonti, M.C., Randolph L.K., and Earle, W.R.: Studies of nutrient media for tissue cells in vitro. VI. The effect of nucleic acid derivatives mixture of medium NCTC 109 on NCTC strain 2071 and NCTC strain 2981. Am. J. Hygiene, 71: 168-175, 1960.

Fioramonti, M.C., Evans, V. J., Mantel, N. and Earle, W.R.: Studies of nutrient media for tissue cells in vitro. VII. The effect of glucuronic acid mixture of NCTC 109 on the proliferation of NCTC strain 2071 and NCTC strain 3145. Am. J. Hygiene, 72: 204-210, 1960.

Menefee, M.G. and Evans, V. J. : Structural differences produced in mammalian cells by changes in their environment. J. Nat. Cancer Inst. 25 1960.

Bakken, P.C., Evans, V. J. Earle, W.R. and Stevenson, R.E.: Establishment of a strain of human skin cells of a strain of human skin cells on protein-free chemically defined medium NCTC 109 in static and in rapidly fluid suspension cultures. Am. J. Hygiene 73: 1961.

Evans, V. J., and Parker, G.A.: The culture of long-term cell strains in protein-free chemically defined medium. Pathologie et Biologie 1961. - This is the publication of the talk given at X° Congres International de Biologie Cellulaire, Paris, Sept. 1960.

Honors and Awards relating to this project:

Continued as member of General and Cellular Biology Review Panel until it was reconstituted as an extra mural panel.

(Evans) Continued as Consultant to Navy Tissue Bank, U.S. Naval Medical School, National Naval Medical Center.

Participated by invitation as discussant of Dr. Theodore Puck's paper entitled "Cytogenetics Tissue Culture", before First Symposium on Methodology on Human Genetics, May 1960, Salt Lake City, Utah. The J. of the National Cancer Institute will publish this symposium.

Presented seminar by invitation to Division of Biology, Goucher College, Towson, Maryland, May 1960.

NCI
Serial No. 428 (c)-Project 2
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 2, Title: Cell transformations in vitro and in vivo.

a) Carcinogenesis potential of tissue culture media.

b) Studies on fresh C3H mouse tissue in chemically defined medium supplemented with some horse serum.

c) Studies on fresh C3H mouse tissue in chemically defined medium with no potential.

d) Studies on transplantability of C3H mouse fibroblasts cultured for a long period in vivo.

Principal Investigators: V. J. Evans and G.A. Parker.

Other Investigators: W. R. Earle and T. A. Keeler.

Cooperating Units: Dr. E. Shelton, Cellular Biology Section, Laboratory of Biology, NCI.

Man Years: (Calendar year 1960)
Total:
Professional:
Other:

Patient Days (Calendar year 1960)

Part B included Yes ___ No. x

Project Description: a) Carcinogenesis potential of tissue culture media.

Objectives: To investigate the possibility of tumor production from injection of 1) the standard chick embryo extract: horse serum medium, 2) Single members of this standard medium mixture, 3) unfiltered chick embryo extract; 4) chemically defined medium NCTC 109, 5) used filtered supernatant fluid from mouse liver cells in vitro.

Methods Employed: All the above media were injected into several hundred NIH Swiss, C3H X AKR, and C3H newborn mice. Injected animals and non-injected controls are being kept one year unless moribundity supervenes. Gross observations were made and representative section of most organs were taken for modified serial section examination.

Results: This is a very long term study because some of the animals have had to be especially bred for the study. A majority of animals, including some of the uninjected controls, have reached the terminal period. The findings as reported last year in the preliminary stage are being confirmed. Anomalies in the lymphatic tissue, kidney, liver and salivary glands and enlarged lymphnodes and spleen were noted.

The earlier finding of heavy infiltration of lymphocytic cells in salivary glands, kidney and liver continue to be confirmed in the Swiss and hybrid strain C3H x AKR. The infiltration also had been seen in the C3H mice in the more recent slides examined. In addition almost all tissue shows varying degrees of perivascular lymphocytic infiltration and this finding seems to occur irrespective of strain of mouse.

Enough tabulations have not been made to give final correlations with any neoplastic changes associated with any specific type of fluid injection or kind of new born mouse.

Significance: Final tabulation of this long term study should contribute baseline knowledge to the phenomenon of cell transformation in vitro and/or tumorigenesis. Resolution of this problem will, it is hoped, contribute to information leading to an answer on why cells transform in vitro. It should also make long term tissue culture strains of additional significance when the findings are correlated to determine if their frequency and nature is significant in relation to the mouse strain injected.

Proposed Course of Project: To continue study until all animals are sacrificed, all autopsies made, all sections obtained and observed, records made, tabulations recorded, correlated and assessed. To determine then the meaning of the heavy lymphocytic infiltration, enlarged spleen and lymph nodes etc., by appropriate bioassay. This appears to be the next problem for experimental examination, even perhaps seeking for a particulate agent in the injected materials.

NCI
Serial No. 428 (c)
Project 2

Project Description: b) Studies on fresh C3H mouse tissue in chemically defined medium supplemented with some horse serum.

Objectives: To determine 1) the time and incidence of transformations that occur in freshly isolated embryonic C3H mouse cells when they are cultured in vitro on chemically defined medium supplemented with horse serum and 2) to record associated phenomena.

Methods Employed: Tissue from C3H embryos was minced and cultured in medium NCTC 109 and 10% (by volume) horse serum. Establishment of cultures in T flasks was carried out by laboratory procedures. Intraocular and intramuscular injections of the tissue were made according to the schedule in the table attached. Cinemicrographic records of tissue cultures of one series have been made periodically over a critical growth period.

Results: The attached table gives the results on the two series reported upon last year as well as the more recent findings. The third series is added as the more recent data not available last year. In all series growth acceleration of the cultures always occurred before any cells could be demonstrated as having been transformed in vitro to produce tumors. In the third series it will be observed tumors occurred after the cells were in vitro only 138 days. This probably is the shortest time that has ever elapsed before mouse cells have been unquestionably demonstrated to have transformed to produce tumors on injection. The interval of time (as determined by the intraocular injection procedure) between when cells will not produce tumors and when they will produce tumors has been determined in this study to be but 14 days. These tumors appear to be fibrosarcomas i.e. the cells in the tumors are elongate, fusiform and some are ovoid with many mitoses. The incidence of tumors is high ranging from 30% to almost 100%. They are transplantable. The third series also confirmed the characteristic behavior, described in last year's report, of the appearance of the tissue cultured cells when implanted in the eye. The latent period of tumors developing in the eye has been as short as 17 days and as long as 125 days. There is some preliminary evidence that it is usually longer when the cells implanted have been in vitro only a short time prior to implantation.

Exploratory chromosome counts made on NCTC strains 3403 and 3631 (Series 1 and 2) after they were in vitro 519 days and 224 days respectively (thus transformed and capable of producing tumors) indicated that there were many heteroploid cells in the populations. The chromosome numbers were hypo or hypertriploid. One cell was observed to be hypertetraploid.

The cinemicrographic studies made on cultures of series 3 for correlation with the data of their sister cultures on the time of production of tumors by the cultures of this series have not yet been evaluated.

Significance: This is probably the shortest time that cells have been cultured in vitro, and it has been subsequently clearly demonstrated that they produced tumors also subsequently shown to be transplantable. It shows that the interval of time when cells in vitro may be considered normal is an extremely short one. The reproducibility of the finding in the three series gives the investigator interested in the transformation of cells in vitro a fairly clear cut calendar for his studies and indicates to him that he has only a short interval of time when he may consider his cells to be normal. It also suggests myriads of problems relating to this very simple finding of the time the cells transformed to produce tumors. Leads on the answers to all must be looked for within four months if the approach is to be a biodynamic one. The data raise such questions as the following: Is the rapidity of transformation of cells (resulting in their being capable of producing tumors) just a peculiarity of embryonic tissue in vitro? Will adult tissue also transform as quickly ? Is this transformation associated with the horse serum ? Is it associated with the small molecule chemically defined medium NCTC 109 ? Is it caused by a "particulate agent" in either of these media introduced on the first day in vitro and requiring a prolonged period to increase in trite in order to yield enough agent to produce a tumor producing transformation ? It will be recalled that an apparent acceleration of growth in the cultures occurs sometime prior to the time that the cells produce tumors on injection. It will also be recalled that the tumors arising from these cultures are apparently fibrosarcomas. Is this acceleration real and is it by any chance associated only with the fibroblastlike cell in the mixed cultures ? Can and do significant numbers of these fibroblast-like cells divide in shorter time i.e. more frequently as the time approached and is finally reached for the cells to produce tumors ? Cinemicrographs already made of the last series of cultures should give an answer to this question. These movies are of cultures whose sisters were used for intraocular implants. If the transformation is associated with growing the cells under anaerobic culture conditions with concomitant alterations in cell metabolism then this short period of growth in vitro is significant as the one in which such a transition has taken place. Since the quantity of tissue is limited in this short period perhaps some consideration should be given to Cartesian diver techniques. Certainly micro enzyme methods to demonstrate changes and, if any are pertinent, also histochemical procedures should be considered. If it is possible that the capacity of the strain of cells to produce tumors on injection is related to a selection of cells in the population, early cloning is essential and confirmation of the data on time of transformation is called for on the cloned strains. Chromosome counts on such cloned populations should be followed through the whole culture period until transformations occur as well as on the embryonic tissue culture system. This has never been done. Data are available on chromosome counts in already transformed tissue, on malignant tissue early in cultivation, and after prolonged cultivation, but this system wherever they can be followed from freshly explanted tissue to transformed cultures is as yet unexplored.

Proposed Course: Prepare for publication pertinent data collected to date. Analyze microcinegraphic records. A study should be made and is planned to see if this transformation will be obtained in fresh tissue, embryonic and adult, employing chemically defined medium and a serum substitute such as albumin to determine the role of the serum, if any. Studies on cloning cells from fresh tissue as early as possible after it is explanted will be attempted. Effort will continue to perfect chromosome analysis techniques and apply them to these cultures as they transform. Photographic records will be made on all cells prepared for chromosome counts for subsequent analysis as opportunity develops. Efforts will be made to establish some collaborations to pursue some of the hypotheses under significance.

Project description: c) Studies on fresh C3H mouse tissue in chemically defined medium for carcinogenesis.

Objectives: To investigate 1) whether or not C3H mouse fibroblasts grown in chemically defined medium NCTC 109 with no added serum or any supplement will produce tumors upon injection into the strain of animal origin and 2) if so after how long a cultivation time in vitro.

Methods Employed: Tissue from 12- 15 day old C3H mouse embryos was cultured in chemically defined medium with no added serum or any other large molecule supplement. Intraocular injections were made of fresh uncultured tissue and of tissue carried by the routine laboratory procedure for various periods of time. This study is closely associated with that described under 428(c), Project 2 b, concerning transformations of cells in a serum containing medium.

Results: Several series of cultures have been attempted in the chemically defined medium with no supplement. Each has grown a little longer than its predecessor. More success appears to be obtained with young embryos. The rate of growth in vitro of the cells is slow with death occurring and waves of new cells coming out. Cells from these series have been implanted in the eye at 26-211 days in vitro. Resorption of these implants to date occurs.

Significance: It is important that time of cultivation of the tissue in vitro per se is not the factor responsible for cell transformation though this claimhas been made. These cultures in the defined medium are not growing at a comparable rate to those in the prior study. Nevertheless continued implantation as long as cultures are viable is necessary for some control on the prior study and on the question of influence of the chemically defined medium in transformation.

Proposed Course: Further eye implants on cells progressively longer in vitro and on any new strains established as fresh tissue in chemically defined medium are to be made. Exploratory studies must be made on the chromosome numbers etc., of any dividing cells in these cultures for comparison with the chromosome changes taking place in the serum containing cultures.

See Table "Intraocular Implants of C3H Cells In Vitro." at end of Project 2.

Project Description: d) Studies on transplantability of C3H mouse fibroblasts cultured for a long period in vivo.

Objective: To determine 1) if cells cultured in vivo transform to produce tumors on implantation in the anterior chamber of the mouse eye and 2) if growth in diffusion chambers in vivo provides stimulus necessary for easy transition to growth in chemically defined medium free of any added supplement.

Methods Employed: Explants of small pieces of epigastric fat pads of the C3H mouse were implanted intraocularly and also in double millipore filter chambers (porosity HA) which were inserted in the mouse abdomen. These chambers were periodically retrieved and the tissue therein subcultured into new chambers which in turn were reinserted into the mouse. Samples from the chambers at the 6 and 11 and 12th generations (359 and 374 days in vivo respectively)were tested for viability in tissue culture in vitro and were also implanted in the anterior chamber of the mouse eye.

Results: Viability was excellent in the chambers retrieved from in vivo generation 12 when the tissue was tested in vitro but serum still appears to be required. The primary explant tissue placed in the eye still survives but does not increase in amount after 12 months in the eye. Implants into the eye made from generations 6 and 11 (359 days in vivo) also are viable but do not appear to show any progressive growth. In fact, the death of the animal is expected to occur from old age before increase in amount of implanted tissue occurs.

Significance: To date after 12 months in vivo mouse fibroblasts have not been transformed to produce tumors on injection in vitro.This same type of tissue cultured in vitro would be expected to have transformed by now. Though growth in chambers has not provided necessary stimulus for cultivation in medium NCTC 109 and in fact tissue culture growth even in serum only results from frequently subcultured in vivo cultures, it is still possible that adaptation to defined medium can be partly carried out in the millipore chambers with an isologous system.

Proposed Course: Anterior chamber implants from tissue retrieved from in vivo filter chambers to test for tumors indicating transformation will be continued. Tissue from chambers more frequently subcultured than those presently tested in order to get more rapidly proliferating growth will be retrieved and tested. Testing of adaptation conditioning of cells in chambers for future in vitro cultivation in a completely defined medium will be carried on.

Intraocular Implants of C3H Cells in Vitro

Days in Vitro	Series I		Series II		Series III	
	Tumors	T.C. Growth Acceleration	Tumors	T. C. Growth Acceleration	Tumors	T.C. Growth Acceleration
28					--	
37			--			
51			--			
56					--	
73			--			
89					--	
93			--	**		
103					--	
107			--			**
117					--	
121			--			
124					--	
138		**			+	
147			+		+	
148	--					
160					+	
168			+			
176	+					
198			+			
199	+					
213					+	
234	+					
262	+					

** = Approximate time when growth acceleration appeared in tissue culture.

Serial No. 428 (c)-Project 3
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 3, Title: Collaborations in Tissue Culture with other organizations.

a) Growth of human skin cells in chemically defined medium in rapidly agitated fluid suspension cultures.

b) Study on the use of human skin epithelial cells cultured in medium NCTC 109 for assaying human viruses.

c) Study on the use of medium NCTC 109 as a solution on wet dressings in surgery.

d) Determination of relative antigenicity of long term strains of human skin epithelial cells and fresh skin cells; long term strains of monkey kidney epithelium and fresh monkey kidney control cells; ong term strains of mouse fibroblasts and fresh subcutaneous connective tissue.

on

e) Effect of iron/proliferation NCTC strain 2071 cells (Earle's strain L fibrosarcoma cells) and NCTC clone 1469 cells (C3H mouse liver parenchymatous cells.

f) Studies using histochemical reactions in selected tissue culture strains.

g) Studies on standardizing the Coulter counter as a means of counting cells in tissue culture.

h) Studies on the use of human skin epithelial cells in long term tissue culture as aid in stimulating skin defect closure.

Part B included Yes ____ No x

Serial No. 428 (c)
Project 3

Principal Investigator: V. J. Evans.

Other Investigators: J. C. Bryant,
E. L. Schilling,
H. A. Kerr,
T. A. Keeler, Jr.,
W. R. Earle.

Cooperating Units: a) Navy Tissue Bank,
Paul Bakken, HM[2],
Kenneth Stewart, HM[1].

b) Dr. Karl Johnson,
LID, NIAID,

c) Dr. J. Wolstenholme,
Veterans Hospital,
Orange, N.J.

d) Dr. M. A. Fink,
Virus Oncology Section,
NCI.

e) Dr. H. Montes de Oca,
Research Fellow-Atomic
Energy Commission, Argentina.

f) idem

g) idem

h) Dr. Arthur Pappas,
Tissue Bank, Naval Medical School,
National Naval Medical Center.

Man Years (Calendar Year1960)
Total:
Professional:
Other:

Patient years (calendar year 1960)

Project Description: a) Growth of human skin cells in chemically defined medium in static and rapidly agitated fluid suspension cultures.

Part B included. Yes___ No x

Objective: To define and control factors influencing growth of human skin epithelial cells in agitated fluid suspension cultures. Emphasis has been placed on adaptation of this strain of cells to cultivation in chemically defined medium NCTC 109 with no added serum for potential virological research, human tumor research, reparative surgery and radiological research.

Methods Employed: These methods for agitated cultures have basically been described (See Project 2, 428 (b). One new basic difference has been incorporated: Cultures were centrifuged in the culture flask prior to fluid renewals and 80% renewals were made. Population counts in agitated cultures were made by nuclei counts of aliquots.

Results: Static cultures can be grown with complete ease in chemically defined medium NCTC 109. In a routine series they increased 8-fold within one week. As an example one fluid suspension culture increased from 12 million to four hundred million in approximately 80 days with fluid renewals at 3 and 4 day intervals, i.e. a 32 fold increase was obtained before the culture was discontinued.

Significance: This is the first strain of human cells that has been grown in agitated fluid suspension i.e. the first strain of human cells that has grown in any large amounts. It may also be the first human strain (irrefutably so identified - see Serial No. 418) in static cultures that has been cultured in chemically defined medium free of any large molecule undefined supplement. These cell have unusual value to the virologist. They make available a predictable supply of human tissue free of wild viruses present in many primary animal cells. These viruses can confuse the test results and in addition be extremely dangerous to the health of the investigator. In addition this cell medium system makes it possible to exclude the use of mammalian serum.

Proposed Course: Collaborative studies using both types of human skin cultures are in progress. Studies to perfect methods for 1) obtaining replicacy of large cultures and 2) for obtaining sheet-like growth in static cultures are in progress. Improvements in both these areas will enormously enhance the usefulness of the strain in planned studies on production of virus pools, high quality antigens, virus assay and radiation biology protection studies.

Project Description: b) Study on the use of human skin epithelial cells cultured in chemically defined medium with no large molecule supplement for assaying virus infectivity of some 8 groups of virus.

Methods: T-9 flask cultures of NCTC strain 3075 cells of human skin epithelium in chemically defined medium free of any large molecule supplements have been prepared and carried to optimum condition for CPE observations and hemadsorption tests. Cultures have been turned over to Dr. Karl M. Johnson (See report NIAD, Lab. of LID); studies with polio, echo, coxsackie,

mumps, influenza and parainfluenza 1,2,3,4 are in progress. Preliminary rough comparative titrations with other continuous lines and primary tissues are to be made by him as initial step in collaboration.

Results: Exploratory data subject to confirmation and discussion with collaborator.

Significance: Any continued success would indicate that virus pools could be prepared, and high quality antigens might be made with these cells. Problems to the virologist such as wild viruses and other infective agents carried by fresh or primary tissue would be oevercome with this type of tissue and complications attendant to the use of mammalian serum in media could be avoided.

Proposed Course: To confirm exploratory and pursue assays on other viruses is the immediate project. Where viruses do not grow as well in primary cultures attempts will be made to adapt such viruses in these continuous lines for the preparation of virus pools and high quality antigens. Explorations will be made of the use of fluid suspension cultures for viruses suspected as more likely to grow best in agitated system. The use of long term monkey kidney cells in chemically defined medium to back titrate virus not showing CPE in skin cells in which they may propagate will be explored.

Project Description: c) Study on the possible use of medium NCTC 109 as a solution on wet dressings in reparative surgery.

Objective: To determine if NCTC 109 medium will serve in various areas of reparative surgery.

Methods Employed: Surgical dressings were kept wet with medium NCTC 109.

Results: In a telephone report from Dr. James Wolstenholme the statement was made that lesions associated with diabetic gangrene and arteriosclerotic ulcers were healing cleanly and rapidly when dressings were wet with medium NCTC 109.

Significance: It was the opinion of Dr. Wolstenholme and the staff of the Tissue Bank , Naval Medical Center (our collaborators) that the medium had real value in wound healing. This study arose out of an earlier collaboration entered into with Dr. Wolstenholme on testing the medium in dog experiments for its value as an intravenous fluid feeding solution which Dr. Wolstenholme discontinued because excess in medium demands could not be met by us.

Proposed Course: This particular collaboration has no future course at this time. Dr. Wolstenholme requested some 75 L of the medium. It was pointed out to him that such an amount could be obtained only by formally

passing a request through appropriate channels in the Veterans Administration and the National Institutes of Health. No request has been forthcoming. Dr. Wolstenholme finds another project of first importance and this of second importance at this moment. The attractiveness of this project was somewhat dimmed for him by the fact that an unlimited amount of medium could not be supplied continuously without administrative clearance. He does state that he proposes in due time to get back to this problem as it still interests him.

Project description: d) Determination of relative antigenicity of long term strains of human skin epithelial cells and fresh epithelial skin, long term strain of monkey epithelium and fresh monkey kidney liver cells, long term strains of mouse fibroblasts and fresh subcutaneous connective tissue.

Objective: To determine if species antigenicity is altered after prolonged cultivation in vitro in vitro at a rapid rate of proliferation and secondarily to determine the significance of such information in classifying and characterizing cells in vitro.

Methods Employed: Several grams of cells of human skin, monkey kidney epithelium and C3H mouse cells 2071 and 929 from Earle's strain have been prepared according to the long term large scale tissue culture procedures of the laboratory (either T-60 flask cultures or agitated fluid suspension cultures). Cells were cropped from flasks, washed and centrifuged and cell pack was made available to Dr. Fink for specialized immunological tests. (See Serial No. 415) Fresh monkey kidney cortex cells were prepared by standard Trypsin procedure; fresh subcutaneous mouse fibroblasts were prepared from C3H mouse connective tissue from site of Earle's original strain L cells.

Results: Results to date indicate that the monkey kidney strain of cells is unequivocally monkey in origin, and also that the human skin cells are unequivocally human. Monkey kidney cells and human skin epithelial cells through prolonged cultivation in vitro still retain antigens of the fresh tissue of the type of their origin. (See NCI 415(c) for more details on antigenic behavior of both fresh and cultured cells)

Significance: Two strains of cells in the laboratory known to have monkey origin and human origin and so maintained in culture to avoid any possibility of species contamination have been confirmed by hemagglutination tests to be monkey and human in origin. Besides confirming that the strains are uncontaminated with other cell species and so making them of unique value for certain biological studies as well as future candidates for tissue culture cell banking, the study has been useful in confirming the value of the test as a means of establishing species identification for any other cell cultures requiring such identification. This is sorely needed since

confusion of cell strains can and has led to presentation of erroneous findings on metabolism studies, virus spectra and nutritional requirements of cell strains. To date no human viruses has been clearly indicated as giving rise to human tumors. Information from further studies in this area may make these long term strains significant tools for human tumor-virus research. If histocompatability antigens are affected tissue cultures containing tumor viruses of human origin might be studied in experimental animals. Basic information on antigenic changes in cells in vitro might lead to suggestions for future rise of cells for clinical application.

Proposed Course: Details indicated on Dr. Fink's report (Serial No. 415) Alterations in both the cells and recipients will be attempted to achieve objective.

Project Description: e) Effect of iron on proliferation NCTC strain 2071 cells (Earle's strain L fibrosarcoma cells) and NCTC clone 1469 cells (C3H mouse liver parenchyma cells).

Objective: To determine if additional iron will improve the chemically defined medium so that it will successfully propagate certain refractory cell types.

Methods Employed: Iron citrate, iron sulfate, iron nitrate, controlled with sodium citrate at 5 different concentrations was added to medium NCTC 109 used as the nutrient solutions for T 15 replicate cultures of mouse fibroblasts and C3H mouse liver parenchyma cells. Growth at end of 10 days was determined by enumeration of nuclei.

Results: No or non-significant growth increase was observed at any of the concentrations. When a concentration of iron was employed at 0.73 mgm 1100 ml. of NCTC 109 it was highly toxic. Analyses of medium NCTC 109 indicated 190 micrograms per 100 ml. of medium as trace contamination.

Significance: This study was carried out as part of the training of a visiting research fellow. Contrary to data reported by another investigator before Tissue Culture Association Meeting in the spring of 1960, no stimulation was obtained from the iron supplement. It is possible that the disparity in results between the two laboratories arises 1) from two methods of measuring culture growth; (nuclear count was used in this laboratory while in the other laboratory hematocrit measurement, method was used) or 2) that the traces of iron already needed by cells are present as contaminants in the medium in the NCI laboratory. The other investigator has no data on amount of iron non-contamination.

Proposed Course: Data suggest it is necessary to determine cell size variation through growth cycle under control conditions and experimental conditions to see whether the test material or the period of the growth cycle of the cultures at which measurements were is responsible for the disparity in the results.

Project Description: f) Studies using histochemical reactions in selected tissue culture strains to determine chemical reactions of different cells in a strain of cells and differences between strains on media variations.

Objective: To determine whether or not 1) cell strains are influenced by the type of nutrient medium on which they are maintained as revealed by histochemical tests; 2) differences exist in cells in a strain.

Methods Employed: Representative laboratory strains of cells were planted in Leighton tubes and cells after growth were fixed with selected fixatives appropriate to the subsequent histochemical techniques applied.

Results: In exploratory studies two strains of cells were particularly interesting. The HeLa cultures maintained in the laboratory on 1% serum contain some cells that are intensely alkaline phophatase positive and other alkaline phosphatase negative. Strain L cells are P.A.S. positive but the intensity of the reaction was much greater in the 929 line than in the 2071 line, the former in stock horse serum medium, the latter in chemically defined medium.

Significance: This study was carried out as part of the training in the laboratory of a visiting research fellow. In biochemical analyses, quantitatively, HeLa would show an overall gross amount of alkaline phosphatase activity in the whole culture with no discrimination as to which cells were producing it. There is a suggestion that strain L cultures appear to show PAS positive material is related to the composition of the medium on which they are maintained, suggesting some quantitative differences in metabolism under different nutritional conditions. These two simple findings, suggest areas requiring biochemical confirmation and also possibilities of use of histochemical techniques in classifying and characterizing cells, when they are adequately confirmed where necessary by biochemical analyses.

Proposed Course: In the hands of a histochemist there is material here for examining the reasons for the two types of cells in the HeLa strain and to determine if the result is a function of the nutrition. Identification of the PAS positive material in strain L might also be studied, since it is not glycogen as it is not digested by diatase.

Project Description: g) Studies on standardizing the Coulter counter as a means of counting all types of cells in tissue culture.

Methods Employed: Cell suspensions, both fixed and unfixed, were photographed and the diameters measured to determine the size of aperature to be used for setting lower threshold.

Results: Ranges in size determined for strain L cells, monkey kidney epithelial cells, human skin epithelial cells, mouse liver cells, high and low tumor mouse cells, and HeLa cells on all the various media on which they are cultured.

Significance: This study is part of the training of a visiting research fellow. References in literature on the use of Coulter counter for counting tissue culture cells indicate no clear evidence that the selected thresholds were set to include all the cells of all sizes. If as appears likely in these earlier studies they have included all cells there is still the question of whether the threshold is just a little too low and non-cellular particles are being counted. Other investigators avowedly determined their thresholds by observing impulse heights in the screen rather than resorting to accurate sizing procedures. Completion of this study will place in the hands of investigators in the field for the first time methods whereby the machine can be standardized for any tissue culture cell type any investigator wishes to use.

Proposed Course: The machine will be used regularly for cell strains that have been validated with some reservations on validity of counts. Counts may be in slight error because of uncertain effect of the disaggregating agents on the cells. Effects of these agents will be explored.

Project Description: h) Studies on the use of human skin epithelial cells in long term tissue culture as aids in stimulating skin defect closure.

Objective: To determine whether or not human skin epithelial cells in long term tissue culture might have any practical value in reparative surgery.

Methods Employed: Human skin epithelium in long term tissue cultures was cultured on a regenerated collagen film as the matrix. Ten to 15 week old BALB/C mice 36 to a group had standard full thickness skin defects created on their backs. Four groupings of mice were made. They received the following treatment 1) Non cortisone treated mice with no material in defect; 2) Cortisone treated mice with no material in defect; 3) Cortisone treated mice with regenerated collagen film in defect; 4) Cortisone treated mice with regenerated collagen film and tissue culture cells in their defects. Six mice in each group sacrificed on 3,6,8,10,13, 16 days post surgery. Standard photographs were made at time defect was created and at sacrific dates. Planimetry determinations were made on photographs. Histological evaluations of defects were made.

Results: The defect closure rate was markedly less in cortisone treated animals than in the non-cortisone treated animals. Application of the collagen film to cortisone treated mice yielded a defect closure rate equal to that of the non-cortisone treated mice. Application of the collagen film with the tissue cultured cells growing on it increased the closure rate of cortisone treated mice when they were compared to their non-cortisone treated counter-parts.

Significance: Apparently the collagen film overcame the inhibitory effect of intramuscular cortisone on the skin defects. Apparently tissue culture cells of human epithelium on collagen film accelerates the rate of healing in cortisone treated mice. The study indicates a fruitful test system for evaluating effect of tissue culture cells on the closure of skin defects. This may be a minor contribution toward the use of a biologic dressing for denuded wound areas in critical situations.

Proposed Course: Manuscript in preparation.

Serial No. 428(d) -Project 1
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 1, Title: Studies of Cell Metabolism in vitro.

Principal Investigator: B. B. Westfall.

Other Investigators: E.V. Peppers, V. J. Evans, K.K. Sanford, H. Montes de Oca, J.C. Bryant, W.R.Earle, E. L. Schilling, L. Dupree and A. Covalesky.

Cooperating Units: NIAMD, Laboratory of Biochemistry and Metabolism, (G.M. Tompkins); NCI, Laboratory of Biochemistry, (E.L.Kuff), (V. Price and R. Greenfield).

Man Years (Calendar Year 1960)
Total:
Professional:
Other:

Patient Days (calendar year 1960)

Project Description: As noted in previous reports the work contained herein is part of the continuing problem-study of the cellular origin of cancer. The work as outlined has several aspects; 1) Studies on the constituents of the medium such as glucose and amino acids removed from the medium during growth of the cells; 2) Studies on materials added to the medium by the cells during their growth in it; 3) Studies on certain enzyme activities of the cells after long cultivation in vitro, e.g., catalase; 4) Studies on lipid content of cells in long term culture with and without lipoproteins present in the medium; 5) Studies on the glycogen content of the cells; 6) Studies on DNA and RNA in the cells after long cultivation.

Part B included Yes ___ No x

Objective: The objective is to learn whether significant differences exist between 1) Cells of different origin; 2) Cells grown under different conditions; 3) Normal and tumor-producing cells; 4) Cells of different tumor producing capacity; 5) Cell strains that have become malignant in vitro, and their capacity to maintain their initial rate of tumor production.

Methods Employed: Cells and medium from the long-term cultures of the Tissue Culture Section are used in all these studies. Adaptation and modification of existing analytical methods or invention of new ones are required for practically all procedures. Validation of such methods for the particular problems requires considerable expenditure of time.

Proposed Course of the Work: Great emphasis will be put on the continuation of the studies concerned with materials removed from the medium and those put into the medium by the cells. Significant results should be attained from the unique cell strains which have been carried for long periods of time in medium to which no protein has been added. These should be particularly significant in aiding the program on simplication of the completely defined medium as well as suggesting leads in designing defined media for cell strains which so far have not been grown in any such defined media. Such studies will entail the use of tagged materials using radioactive preparations. This work will be unique because metabolism studies in vitro to date have been confused by not having been done on long term strains cultured in the absence of added protein. Glycine and serine for example increase growth rate of cells; they might therefore be incorporated into thenucleic acids of the cells. Whether or not this is so needs to be determined. Many investigators using serum in the medium have said glycine and serine are not essential in the medium; so this controversial point on the function of these amino acids assumes significance. This and similar studies will be approached through the use of radioactive isotope tracers. Also since cytidine and thymidine are included in the chemically defined medium, studies are now being planned to investigate the possible role of B12 and folic acid in their utilization of these two nucleic acid derivatives by means of tritium tagging.

With an increased number of clone strains of cells now able to grow in chemically defined medium free of added protein, and with increased and increasing simplification of this medium, plans are now being made to continue study along the indicated lines.

Major Findings: Sub-project A: a-Keto acids in the medium as a result of the cells in a medium originally containing no keto acids. Eleven mammalian cell strains, 9 from the mouse and the other two of human origin were used in the study. All were long-term strains. They were all able to put into the medium appreciable quantities of pyruvic and alpha-keto-glutaric acids indicating that all of them possessed the transaminating enzymes

needed to form these compounds from the corresponding amino acids, or, in the case of pyruvic acid formed it from glucose. Using the 1469 strain of mouse liver cells it was demonstrated that the enzymes concerned with formation of alanine from pyruvic acid and of glutamic acid from the a-keto-glutaric acid were still present in the cells after long growth in vitro.

Sub-project B: Note on the catalase activity of several mammalian strains after long cultivation in vitro. Twelve long-term mammalian tissue cell strains were examined for their catalase activity. These included three strains developed from parenchymal epithelial tissue of the mouse liver. All strains had very low activity. The tumors rising from injection of some of the strains deriving from mouse tissues into mouse likewise had low activity. The hepatomas rising from injection of the hepatoma strain of mouse cells likewise had low activity. Contrary to general impressions from the literature the presence of these hepatomas in host did not greatly depress the catalase activity of the liver of the host. Comparison of the activity of the host's liver with the catalase activity of the liver of a mouse bearing a spontaneous hepatoma indicated that the presence of the hepatoma in the liver did not greatly depress the activity of either the liver or the hepatoma.

Significance: The studies indicate that there is no necessary parallelism between the behavior of various enzyme systems in cells grown for long terms in culture. In the one instance (Catalase) there was very great reduction in the activity of the enzyme in the cells originating from liver and in the other (transaminating enzymes) they were still highly active. This probably means that under these conditions of growth catalase has very little use-fulness to the cells and is largely lost, whereas transaminases still are needed in abundant quantity.

Part B Honors, Awards and Publications:

Publications:

Westfall, B.B., Peppers, E.V., and Earle, W.R.: a-Keto acids in tissue culture. II. Studies on the used medium. J.Nat. Cancer Inst. 24: 417-423, 1960.

Peppers, E.V., Westfall, B.B., Kerr, H.A., and Earle, W.R.: Note on the catalase activity of several mammalian cell strains after long cultivation in vitro. J. Nat. Cancer Inst. 25: 1065-1068, 1960.

Honors and Awards: None

NCI
Serial No. 428(e)-Project 1
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 1, Title: Some effects of vitamins on cells cultured in a protein-free, chemically defined medium.

Principal Investigator: K.K. Sanford.

Other Investigators: L.T. Dupree, A.B. Covalesky, W.R. Earle.

Cooperating Units: None.

Man Years (Calendar Year 1960)
Total:
Professional:
Other:

Patient Days(calendar year 1960)

Project Description: Objectives: A protein-free chemically defined medium NCTC 109 developed in this laboratory has been found satisfactory for long-term proliferation of several cell strains from three different mammalian species, including a derivative of strain L mouse cells designated cell strain 2071. Based on quantitative experiments by Dr. V.J. Evans and associates, a simplified medium designated NCTC 118 has been developed for strain 2071 cells that contains a group of amino acids, 2 nucleic acid derivatives, sodium acetate, glucuronolactone, glutathione, salts of Earle's saline, and 18 vitamins. One objective of this project is to analyze the effect of these 18 vitamins, individually and in different combinations, on the survival, proliferation, morphology, and fragility of strain 2071 cells, assayed at known inoculum levels as suspensions of single cells or small cell clumps. A second objective is to simplify medium NCTC 118 by eliminating all vitamins not required by the cells for rapid

Part B included Yes ___ No x

proliferation and thus to define the vitamin requirements of the cells in the absence of added serum proteins, peptones, or other supplements which introduce unknown and undefined nutritional factors. A third objective is to evaluate the interrelationships of B_{12}, folic acid, and thymidine for cell proliferation. A fourth objective is to isolate any nutritional variants from the cell population of this clone that can be used in studies requiring cells with biochemical "markers".

Methods Employed: Quantitative replicate culture procedures developed in this laboratory have been used to determine growth responses of the cells to each vitamin. A period of at least 2 months was usually found necessary for vitamin depletion before the growth response to the added vitamins could be quantitatively measured. Effects on cell morphology were evaluated from photomicrographs of the living cells. Fragility tests based on the response of cells to mechanical stirring of cell suspensions have been used.

Major Findings: As outlined in last year's report, when each of the 18 vitamins was individually deleted from the medium, 5 vitamins, namely calcium pantothenate, choline chloride, niacinamide (or niacin), thiamin, and riboflavin were identified as essential for cell survival. Vitamins not apparently required for cell survival but which increased rates of cell proliferation were folic acid and pyridoxal (or pyridoxine).

During the past year, a new medium NCTC 119 containing only these 7 vitamins was evaluated.

The new medium NCTC 119 containing only 7 of the 18 vitamins failed to support cell survival for more than 2 or 3 weeks. The following additional vitamins were tested singly and in combination: B_{12}, p-aminobenzoic acid, biotin, pyridoxine, and niacin. The data showed the importance of biotin, a requirement which could not be demonstrated earlier in the presence of the 18 vitamins.

In the new medium, NCTC 120 containing biotin, repeated tests showed that the cells though proliferating as rapidly as the controls in medium NCTC 119 were fragile and could not withstand the stirring used to set up quantitative experiments. A test for fragility was then developed and standardized. At the present time, eleven vitamins are being returned to the medium singly and in various combinations in an effort to detect what ones are necessary to prevent fragility of the cells.

The interrelationships of vitamin B_{12}, folic acid, and thymidine in their effects on cell proliferation and growth were investigated. When deleted individually, these components had no detectable effect on cell survival

or proliferation. When all 3 were deleted, growth was poor and enlarged cells resulted with abnormal nuclei. In the absence of folic acid and B_{12}, there was some reduction in cell growth. A more marked reduction in growth occurred when thymidine and folic acid were removed from the medium. In the latter medium, containing only B_{12}, cells increased in nuclear mass and cell numbers decreased to a critical point; then the few remaining viable cells seemed to become adapted to the medium and start dividing, nuclear mass decreased and the cells resumed a healthy appearance showing a high proliferation rate. After a period of 15 to 25 days cells reverted to the original condition and failed to survive. In medium containing thymidine and folic acid, there was some evidence that vitamin B_{12} influenced the survival of small numbers of cells, though this point is being investigated further.

One nutritional variant isolated in this study has been compared with the parent line. Cells of this variant line do not require pyridoxine or pyridoxal for rapid proliferation as compared with the parent line. However, the cells are exceedingly fragile.

Significance: Although others have attempted to define the vitamin requirements of strain L cells in media containing added serum proteins or other undefined supplements, the system developed in this laboratory free of added proteins and also antibiotics which could favor intra cellular bacterial growth, is unique for accurate studies of vitamin requirements and interrelationships. Although others have suspected the importance of biotin, to our knowledge this is the first demonstration of its importance in tissue culture. Also, the effect of vitamins on the fragility of the cells has not been demonstrated or investigated so far as we know. These studies are significant as a final step in simplifying medium NCTC 118 for further comparative studies on nutritional requirements of cell strains of normal or neoplastic tissue origin. With such a completely defined medium, reduced to only those components the cells actually require, the role of each of the components in intermediary metabolism can then be examined under rigidly controlled conditions.

Proposed Course of Project: This project will be completed to obtain the objectives listed.

NCI
Serial No. 428(e) -Project 2
1. Laboratory of Biology,
2. Tissue Culture Laboratory

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project 2, Title: Clonal studies and cell transformations in vitro.

Principal Investigator: K.K. Sanford,

Other Investigators: L.T. Dupree, A.B. Covalesky, B.B. Westfall, and W.R. Earle.

Cooperating Units: H.B. Andervont, Laboratory of Biology,
E.L. Kuff, Laboratory of Biochemistry,
T.B. Dunn, Laboratory of Pathology,
D.B.McN.Scott, University of Pennsylvania.

Man Years (Calendar Year 1960):
Total:
Professional:
Other:

Patient Days (calendar year 1960)

Project Description: This project consists of a number of separate studies which will be considered under the above heading. Study 1. Cloning of mammalian cells by a simplified capillary technique. A manuscript describing this cloning procedure, and including an historical review and evaluation of other cloning methods has been prepared and is in press. See attached abstract.

Part B included: Yes X No ____

Study 2. Polyoma virus and production of malignancy in vitro. Details of this study were presented in last year's annual report. The study was completed and a manuscript was prepared that is now in press. See attached abstract. Data on susceptibility of seven different long-term cell strains to polyoma will be prepared for publication.

With the development of a susceptible clone of mouse parotid cells, we would like to continue a further cinematographic analysis of the effect of this virus on individual cells of the population. However, our present sterile room facilities are inadequate to pursue this line of work as we would wish since without segregated areas for virus research, contamination of our other cell strains would be difficult to avoid.

Study 3. Analysis of enzymatic activities of clones derived from variant cell lines transformed to malignant cells in tissue culture. This collaborative study with D.B.McNair Scott has been written up for publication and is in press. See attached abstract.

Study 4. Sarcomatous transformation and persistence of the mammary tumor agent in long-term cultures of mouse mammary carcinoma. This project described in last year's annual report was completed, and a manuscript prepared that has been submitted to the Journal of the National Cancer Institute. See attached abstract.

Study 5. Alterations within a clone of mouse tumor cells. This study of morphologic and enzymatic alterations within a clone of cells has been completed and a manuscript prepared that has been submitted to the Journal of the National Cancer Institute. See attached abstract.

Study 6. A clonal study of mouse pulmonary carcinoma. Clones of pulmonary carcinoma cells have been followed with respect to maintenance of differentiation and sarcomatous change both in vitro and when returned to the mouse and grown as tumors. The results of this study are now being analyzed.

Study 7. With Dr. Du Buy and also Dr. Kuff, orientation experiments have been carried out on induction of cell change by transfer of subcellular particles.

Proposed Course of Project: During the past few years we have studied many types of transformation, i.e., neoplastic, enzymatic, morphologic, and chromosomal occurring within clones of mammalian tissue cells. We now wish to study the mechanism of these changes and attempt to control and induce them. An experiment is being planned to test the role of anaerobiosis as a factor in the neoplastic transformation of normal tissue cells in vitro. As a result of Westfall's identification of biochemical markers and our identification of nutritional variants, we are in a position to test for

transfer of genetic materials between cells. About a year ago, our orientation experiments in this area were unsuccessful, but the recent success of Barski, Sorieul, and Cornefert using two of our clones in demonstrating "hybridization" of cells as evidenced by chromosome markers stimulates us to further efforts in this area. First, we will try to confirm the observations of these workers. Several other approaches will then be used in an effort to induce and control transformations within clones.

Abstracts of Manuscripts in Press:

Cloning of Mammalian Cells by a Simplified Capillary Technique. By: K. K. Sanford, A. B. Covalesky, L. T. Dupree, and W. R. Earle. A method is described for the critical isolation of clones from single mammalian tissue cells in culture. Early attempts to isolate and grow single cells and various cloning methods are reviewed.

Polyoma Virus and Production of Malignancy in Vitro. By: K.K. Sanford, T. B. Dunn, A.B. Covalesky, L.T. Dupree and W.R. Earle. An attempt was made to induce a rapid and reproducible neoplastic conversion in a single-cell clone of mouse cells during the period in vitro before "spontaneous" conversions occur. Such changes were repeatedly produced in a clone derived from the salivary gland of a C3H mouse by infection with polyoma virus. The mechanism of this change, whether by cell selection or by cell change, awaits further study. Cells of the salivary gland clone were also compared with 2 other long-term strains of C3H mouse cells, one derived from minced whole embryos and the other from adult connective tissue. Cells were compared with respect to viral susceptibility and tumor-producing capacity. Of the 3 cell types, only the adult fibroblasts from connective tissue failed to show any cytopathogenic response to infection with polyoma. It was shown further that all three cell types cultured in a serum-supplemented chemically defined medium, will ultimately undergo a malignant conversion in vitro when no virus is purposely added. Tumors produced by the salivary gland clone were extraordinary because of the amount of collagen they contained and the distortion of the leg they produced when cells were transplanted to the thigh muscles.

Analysis of Enzymatic Activities of Clones Derived from Variant Cell Lines Transformed to Malignant Cells in Tissue Culture. By: D.B. McN. Scott, A. M. Pakoskey, and K.K. Sanford. Two cell lines derived in vitro from one cell isolated from a culture of normal mouse tissue were found in previous studies to differ in morphology, metabolism, and in their rate of growth to form malignant tumors in mice of the strain of origin (C3H). When cells of one line, designated low tumor-producing or "low" were injected into mice, tumors developed slowly or not at all, whereas tumors developed rapidly from implants of the other cell line, designated high tumor-producing or "high". Three clones established from single cells of the low line and one clone from the high line have been investigated to determine whether there is any correlation between activities of some of the enzymes metabolizing glucose oxidatively and the rate of growth into tumors when cells are implanted in mice. The activities of the enzymes hexokinase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase have been determined in the whole cells and in cell-free extracts of the cells. It was found that the mean specific activities of all three enzymes were very similar for two of the clones from the low line. The third of the clones from the low line that differed in certain other characteristics had the same phosphogluconate activity, a 50% higher hexokinase activity, and twice the glucose-6-phosphate dehydrogenase. The clone derived from the high line

had nearly three times the hexokinase activity, four times the glucose-6-phosphate dehydrogenase activity, and five times the phosphogluconate dehydrogenase activity compared with two of the low line clones. The differences between the derivative clones from low and high lines are even greater when the enzyme activities of the whole cells or the extracts are calculated per cell.

Sarcomatous Change and Maintenance of the Mammary Tumor Agent in Long-term Cultures of Mouse Mammary Carcinoma. By: K.K. Sanford, H.B. Andervont, T. B. Dunn, L.T. Dupree, A.B. Covalesky, G.L. Hobbs, and W. R. Earle. This study had three objectives: 1) to determine whether the mammary tumor agent persists in long-term cell strains cultured from mammary carcinomas of the strain C3H mouse; 2) to follow the behavior of these cells in vitro with respect to maintenance of differentiation as assayed in vivo; and 3) to supply additional evidence regarding the "sarcomatous" transformation which epithelial tumors frequently undergo and which appeared in the cell strains of the present study. Cell strains were established in culture from 18 different tumors of mammary gland origin. In certain cell strains activity of the mammary tumor agent could be demonstrated after 6 months to more than a year of rapid cell proliferation in vitro. In other cell strains, the agent seemed to disappear. The type of culture medium appeared to be an important factor influencing the maintenance of differentiation in the culture strains. One strain grown in serum-supplemented chemically defined medium continued to reproduce in mice the epithelial glandular architecture of the original tumor after 1-1/2 years and 35 transplant generations of growth in vitro. Cells cultured in the serum-embryo extract medium and transferred from culture to mice during the first 5 months in vitro grew as differentiated mammary carcinomas, but cells transferred after this period produced sarcoma-like tumors. Experimental evidence indicated that the "sarcomatous" change resulted from two different phenomena: 1) neoplastic transformation of stromal cells in culture and 2) morphologic alteration of carcinoma cells so as to resemble sarcoma cells of connective tissue origin.

Alterations in Vitro Within a Clone of Mouse Tumor Cells. By: K.K. Sanford, B.B. Westfall, H.Y. Chu, E.L. Kuff, A. B. Covalesky, L.T. Dupree, G.L.Hobbs, and W. R. Earle. The studies describe the development in vitro of hereditary differences in morphology, further alterations in arginase activity, and also apparently B-glucuronidase activity, within a single-cell clone of mouse tumor cells. The difference in arginase activity persisted during long-term culture of the cells in different media in vitro as well as during growth of the cells as tumors in mice. By means of clonal analysis, additional hereditary alterations in morphology and level of arginase activity within the tumor cell clone have been identified. Data are presented on the tumor-producing capacity of the cells, the arginase activity of the tumors grown from the tissue culture cells, the arginase activity of clonal derivatives, the B-glucuronidase activity of the cells, the chromosomal constitution of the cells in vitro, and certain other biochemical properties dis-

tinguishing these cells from some other strains of cells grown for long periods under similar culture conditions in vitro.

Part B Honors, Awards, and Publications:

Publications other than abstracts:

Scott, D. B. McN., Pakoskey, A.M., and Sanford, K.K.: Analysis of enzymatic activities of clones derived from variant cell lines transformed to malignant cells in tissue culture. J. Nat. Cancer Inst. In press.

Sanford, K.K., Covalesky, A.B., Dupree, L.T., and Earle, W.R.: Cloning of mammalian cells by a simplified capillary technique. Experimental Cell Research. In press.

Sanford, K.K., Dunn, T.B., Covalesky, A.B., Dupree, L.T. and Earle, W.R. : Polyoma virus and production of malignancy in vitro. J. Nat. Cancer Inst. In press.

Sanford, K.K., and Westfall, B.B.: Alterations in vitro within a clone of mouse tumor cells. Pathologie et Biologie. In press.

Sanford, K.K.: Discussion of Dr. Puck's paper entitled "Tissue Culture" in the Symposium on Methodology in Human Genetics. In press.

Honors relating to this project: Invitations to present papers at the University of Texas Medical School, M.D. Anderson Clinic from Dr. T.C. Hsu and at the University of Minnesota Medical School, from Dr. J.T. Syverton.

Invitation to participate in symposia on Tumor Viruses and Methodology in Human Genetics. Invitation from Dr. J. Danielli to write a chapter on "Cellular Transformation and the Genesis of Tumor Cells" in the next edition of the International Review of Cytology. Invitation to membership on the Editorial Advisory Board of Cancer Research, and on the Cell Culture Coordinating Committee.

Serial No. 428 (f)
1. Laboratory of Biology,
2. Tissue Culture Section,
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies in tissue culture of the malignant cell and of the normal cell from which it arises. (This is the general title for all work in this Section.)

Principal Investigator: W. R. Earle.

Other Investigators: See 428(a) through 428(e) preceding.

Cooperating Units: Listed under parts (a) through (f).

Man Years: (Calendar Year 1960)
Total: 19
Professional: 9
Other: 10

Patient Days (calendar year 1960)

Project Description: The above project may be considered as the general title for all work in the section; under the present subheading (f) will be outlined a number of activities not included within 428 (a) through (e).

1) Constant temperature rooms have been calibrated and are in routine use. Already certain reductions of experimental error has been accomplished as compared with old studies in incubators. The room will reach their full significance, however, only when panel slots and microscope incubators are completed. Use of the rooms has, as previously noted, already allowed discard of 4 incubators with others to follow on completion of the slots.

Part B included Yes____ No x

2) Detailed studies on the cinematograph have been described under 428(a). Equipment for processing film is now installed, and while needing a few minor shop changes, is in routine operation. Use of this equipment is seriously handicapped by lack of space, however.

3) Precise equipment for more accurate control of gas tensions in tissues is in part completed and in active operation. Other phases of this control of gas tensions, mentioned in last year's report, are under study or development.

4) Check list of publications of this section has been brought up-to-date as of September 1, 1960, and is now available.

5) Additional patents, assigned to the Government have been issued or should shortly be issued. Patents to date include (1) successful growth of single isolated fixed tissue cells (issued); (2) successful growth of fixed tissue cells in rapidly agitated fluid suspension culture (interference won); patent due to be issued shortly; (3) special design of culture flask (T flask) suited for quantitative tissue cultures (issued); (4) special methods devised for fabrication of such flasks (issued).

6) Consultancy of W.R. Earle to the Tissue Bank, National Naval Medical Center has been continued.

7) Consultancy of W.R. Earle to Eli Lilly Research Institute continued.

8) During the last year we continued to have an extremely large number of visitors, many of whom have been from foreign laboratories or who have come through to evaluate the practicability of setting up tissue culture work in foreign countries. Still another group has been primarily interested in our methods which have maintained culture strains over many years free from demonstrable pleuropneumonia-like infections. Practically all visitors have been primarily interested in tissue culture and its applicability in various lines of work. Many have had specific technical inquiries on which we could advise them with respect to difficulties encountered. Others have wanted to see the general design and operation of the procedures and equipment of the section. Some have asked authorization to remain and work here for an extended time in order to obtain training. While we have tried to assist such workers, lack of space and equipment facilities have forced us to limit the time allowed to what a worker could observe in a limited number of hours. In each instance we try to evaluate the needs of each individual visitor or group. There is no doubt, however, that this handling of visiting professional personnel is extremely time consuming. Further, the time taken up is that of the professional staff because very few visitors are of a type whose desire for information can be met by untrained or subprofessional personnel.

9) Work of section included as summary in film of British Broadcasting Company.

10) Insofar as appears to us practical we try to furnish parent cultures to allow other workers and laboratories to start cell strains in their own organization. No cell strains are furnished which are commercially available, or available from other sources, in satisfactory condition. Cell strains are furnished by Tissue Culture Section, NCI, under conditions previously approved by the Director of the National Cancer Institute.

11) Attention should be called to the extreme crowding of the personnel, inadequate subprofessional personnel, and the effect of these conditions on efficiency of the work. As instances, certain cell lines have had to be closed in spite of the fact that they were considered significant. A series of studies with the polyoma virus were initiated and yielded significant results. In these a normal strain of parotid gland cells was transformed in tissue culture by means of the virus into malignant cells. Since adequate room space did not allow safe handling, all work with the polyoma, both in vivo and in vitro had to be closed. No senior investigator has any privacy for research planning, analyzing data, or writing up results. The head of the section often has to discontinue or seriously curtail work in his room during time essential photographic records are being made. In instances laboratory bench space is so limited that actual mixing of solutions has had to be carried out in the hall. Often equipment has to be temporarily rolled out into the hall to make room for certain operations in the laboratories. Rearrangement of the laboratories has been repeatedly attempted but has been handicapped by lack of personnel such that apparatus being rearranged could not even be cleaned up. It has been desired to get rid of certain obsolescent equipment but room and space have seriously handicapped this procedure. Development of new equipment and methods had been seriously handicapped both by lack of space and by present systems of shop facilities.

With all of these handicaps it is the feeling of the senior personnel of the Section that the past work within the section has opened up unique and extensive new potentialities for further advance in the application of long-term large scale tissue cultures in the study of the malignant cell and of the normal cell from which it arises, and for further exploration of this transition.

12) The cultures within this section have been carried aseptically, with an absolute minimal and usually no use of antibiotics. Recent collaborative studies of our cultures has shown all cultures maintained in the section are apparently free from pleuropneumonia and diptheroid organisms which have so seriously complicated the interpretation and evaluation of nutritional, biochemical and other biologic data from laboratories relying on antibiotics. To our knowledge, this is the only large scale, long-term tissue culture laboratory whose experimental results have not been complicated by such contaminations. The seriousness of such contamination can

invalidate experimental studies to such an extent that the presently proposed repository for tissue culture cells, now under planning by the American Type Culture Collection, calls for demonstration of the absence of such contaminants from culture strains submitted for the respository. It is also of interest that clone NCTC 929 has been requested as strain No. 1 for this repository.

Part B Honors, Awards and Publications:

Publications - as listed in sections 428(a) through (e)

Honors:

Requested to introduce, act as Chairman and summarize symposium on tissue culture, at Rutgers University, February 1961.

Requested to organize and serve as Chairman of Symposium on Growth of Cell Cultures in Vitro for Fermentation Subdivision, Division of Agricultural and Food Chemistry, American Chemical Society, September, 1961.

Serial No. NCI-410
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Maryland

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Individual Project Report
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Part A

Project Title: Biological and biochemical studies on a murine lymphoid leukemia virus originally recovered from Sarcoma 37.

Principal Investigator: J. B. Moloney

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960
Total: 4
Professional: 2/3
Other: 3-1/3

Project Description:

Objectives.--A comprehensive study of the biology and biochemistry of the leukemia virus with a view toward an understanding of the nature of the agent and its relation to the neoplastic process.

Methods Employed and Major Findings:

Biological.--This virus produces a generalized lymphocytic neoplasm in 100 percent of the recipients within a relatively short period when inoculated into mice of various ages and strains and into rats. The disease produced is similar to that which occurs spontaneously in certain inbred strains of mice.

1. Sources of the Leukemia Virus: The agent was originally recovered from Sarcoma 37 which had been transferred to BALB/c mice after 195 tumor generations in strain A/Ln mice. Sarcoma 37 is maintained as a routine passage series in BALB/c mice and still yields the virus after more than 100 transplant generations. Of particular interest is the finding that other lines of Sarcoma 37 are also carrying the virus. Thus, the original tumor in A/Ln mice and the Sarcoma line from which this arose, and which is now being carried in ZBC mice by the Laboratory of Chemical Pharmacology and Microbiological Associates,

have been extracted and assayed for leukemia virus content. The incidence of leukemia and the latent periods in the inoculated hosts were found to be similar to those which received virus concentrates derived from the BALB/c Sarcoma 37.

Additional sources of the virus are as follows: (1) leukemic lymph nodes, thymus glands, and spleens from mice which had received cell free concentrates of Sarcoma 37; (2) leukemic hematopoietic organs from mice which had received whole cell intraperitoneal implants of leukemic tissues, originally derived from cell free concentrates; and (3) lymphosarcomas (localized leukemias), both in the solid and ascites form, induced by the subcutaneous and intraperitoneal inoculation of virus-induced neoplastic cells.

2. Recovery of the virus from the source tissues: The techniques used for the recovery and concentration of the leukemia virus from the source tissues have been illustrated and described in detail in previous publications (see also Part B of this report). In addition to the standard procedures of centrifugation alone or in combination with filtration for the production of crude microsome fractions containing the active virus, we have investigated a physiochemical procedure originally developed for the partial purification of the Rous sarcoma virus. This procedure which precipitation, followed by trypsin digestion of the protamine-virus complex, results in a leukemia virus preparation of high biological activity and of a high degree of purity on a unit of nitrogen basis.

3. Routes of inoculation: The leukemia virus is effective in mice when administered by any one of the following routes: subcutaneous, intracerebral, intraperitoneal, intravenous, and oral. Regardless of the route of inoculation, the neoplasm produced is always of the generalized type No tumor appears at the site of inoculation. In weanling and adult mice the intravenous route is the most effective. On the average mice inoculated intravenously die with leukemia one month earlier than those receiving virus by other routes.

4. Host specificity: The host specificity of the leukemia virus has been investigated with respect to age, strain, and species.

Age.--It was found that the virus is capable of eliciting a 100 percent leukemia response in BALB/c mice inoculated as late as 8 months of age.

Strain.--The virus is not mouse strain specific. All inbred strains and hybrid groups of mice tested in this laboratory have reacted to the virus with a leukemia incidence level of essentially 100 percent. Included in these experiments was a group of C57BL mice which developed leukemia in all cases but after an extended latent period. This finding indicates a possible degree of resistance in the C57BL strain to the leukemia virus.

To date no strain of mouse has been found to be completely refractive to the leukemia virus. In this respect Dr. Yoshihiko Tsubura of the Nara Medical College, Japan has found that newborn and adult mice of the Japanese inbred dd strain (a normally low leukemic strain) are highly susceptible to leukemia induction by the virus (personal communication).

Species.--In view of the apparent lack of age and strain specificity of this leukemia virus, the host studies were extended to include animals of other species. Sprague-Dawley and Osborne-Mendel rats less than 36 hours of age were inoculated with the agent derived from a pool of virus-induced mouse leukemic thymus glands and lymph nodes. The rats received the virus intracranially, intraperitoneally or subcutaneously. All (100 percent) of the inoculated rats died with a generalized lymphoid leukemia within an average latent period of approximately 14 weeks. A comparative assay of a 10^{-1} dilution of the same mouse virus preparation in newborn BALB/c mice produced leukemia in 100 percent of the recipients, with a mean-time-to-death of 15.9 weeks. Thus, although the data are somewhat limited at this time, no highly significant difference in the susceptibility of mice and rats to the same virus preparation is apparent.

The recovery of virus from the induced rat leukemia tissues has been demonstrated by the passage of cell-free extracts of this material in newborn rats and also in mice. Although the third rat "selective virus passage" experiment is still in progress, both rats and mice are dying with leukemia as early as 7 weeks after inoculation of the rat derived virus preparation. The leukemia agent, therefore, has not acquired host specificity after repeated virus passage in the rat or the mouse. The gross pathology of the induced disease in rats is discussed below:

It is of interest to note that the virus induced rat leukemia is also freely transplantable within the strain of rat. The neoplastic cells, when inoculated subcutaneously in adult Osborne-Mendel rats, produce a localized leukemia lymphosarcoma at the site of implantation.

These studies describe for the first time: the viral induction of lymphoid leukemia in the rat; a lymphoid leukemia virus which can cross the species barrier; a virus which is not age and strain specific in the mouse; a virus which is not rat strain specific.

5. Serial virus passage in mice: In an effort to increase the potency of the leukemia virus, studies were undertaken which employ the techniques used to increase the concentration of virus in source tissues in virus-induced avian neoplasms. The details and operative procedures are described in previous reports (see also Section B of this report).

The results to date, based on latent period data, indicate that the potency of the virus has been increased with successive passages. The first passage material induced lymphocytic leukemia in 100 percent of the test animals, with an average latent period of 6.4 months. After 12 virus

passages in newborn mice, the agent now induces the generalized neoplasm in a high percentage of animals as early as 5.5 weeks after inoculation. One hundred percent of the inoculated mice have leukemia within a latent period of 2.5 months.

In these studies, latent period is defined as time from virus inoculation to palpable inguinal lymph nodes and/or spleen, a response criterion employed in the biological assay of the agent. Time-to-death after detection of infiltrated organs varies from 11 to 28 days, with a mean of 19 days.

6. Pathology of the induced disease: In mice.--The macroscopic and microscopic pathology of the disease in this species has been completely described in previous reports. The pathogenesis of the virus induced leukemia is described in NCI-410 (a).

In rats.--Macroscopic: Over ninety percent of the highly inbred Osborne-Mendel rats with the induced disease exhibited the characteristics of a generalized lymphocytic neoplasm that involved the spleen, liver, lymph nodes and thymus. Autopsy of the Sprague-Dawley rats (a poorly inbred line) disclosed that the leukemia was limited to a marked enlargement of the thymus gland. The average weight of these infiltrated thymus glands was 7.0 grams (normal thymus 0.5 grams).

Microscopic: Histologic studies on rats with the generalized disease showed the neoplasm involved many organs and tissues. Infiltration by the malignant cells destroyed the normal architecture of the thymus and involved lymph nodes, and extended beyond the boundaries to involve adjacent organs and tissues.

7. The effect of splenectomy or thymectomy: The effect of splenectomy and/or thymectomy on the viral induction of leukemia in adult mice is being studied. Five to eight week old BALB/c mice were inoculated with the lymphoid leukemia agent 48 hours following the operation. The results to date can be briefly summarized as follows: Either thymectomy or splenectomy delays the onset of leukemia in the inoculated hosts although all eventually die with the disease. Thymectomy of the host followed by the inoculation of the lymphoid agent results in the induction, in a high percentage of the cases, of a Hodgkins-like lesion which is a reticulum cell neoplasm of Type B as described by Dr. Thelma Dunn.

Splenectomy of the host followed by the inoculation of the lymphoid agent results, in a high percentage of the cases, in the induction of myeloid leukemia which can be further differentiated as chloro-leukemia. This myeloid leukemia is now well established in the 12th whole-cell passage in BALB/c mice and still maintains its chloro-characteristics. It should be emphasized that this lesion is extremely rare in this strain of mouse. We have extracted and concentrated the induced myeloid leukemia and inoculated the cell-free material into intact newborn BALB/c mice. All of the mice died with lymphoid leukemia. It is particularly interesting,

therefore, that a single virus entity can induce one of three neoplasms of the reticulum system of the host, depending on the available substrate, or condition of the recipient.

Studies are continuing in this area to determine (1) effect of splenectomy and/or thymectomy at various time intervals following virus inoculation, (2) effect of reimplantation of thymus and/or spleen (as well as cell free extracts of these organs) at various time intervals following thymectomy and/or splenectomy and virus inoculation.

8. Immunological studies: Collaborative studies on the antigenic nature of the leukemia virus are described in NCI-410 (a). In addition to the homologous antiserum described therein, we have been successful in producing a heterologous antiserum in rabbits which effectively neutralizes the leukemia virus. Although the absolute titer of the antiserum remains to be determined the findings indicate that a final dilution of 1: 10 of this antiserum completely neutralized a $10^{-2.3}$ dilution of leukemia virus. Rabbit anti-normal spleen extract serum and normal rabbit serum had no effect on the leukemogenic activity of the same virus material.

Experiments designed to demonstrate an immunological relationship (if any) to other known leukemia viruses are in progress. Rabbit antisera known to be effective in neutralizing the Gross virus and the Friend leukemia agent (these sera were very kindly supplied by Dr. Gross and Dr. Friend) are under test to determine their neutralizing effects on the leukemia virus described herein. Although the experiments are still in progress, to date there is no indication that either antiserum inactivates the leukemia virus recovered from Sarcoma 37. It remains to be determined if this this is simply a quantitative effect.

Biochemical.--

1. The results of preliminary investigation into the biochemical nature of an active virus preparation are given below:

Analysis	Mg/ml
Total N	0.46
Total P	0.07
Pentose Nucleic acid	0.27
Deoxypentose Nucleic acid	0.00

The value (0.07 mg/ml) given for total phosphorus reflects the lipide-, nucleic acid-, and protein-phosphorus content of the particulate suspension.

With regard to the nucleic acid content, no deoxypentosenucleic acid (DNA) was found as measured by the diphenylamine reaction. The absorption of the nitrogenous bases at 2600 Å in perchloric acid extracts of the particulate material was entirely accounted for by the amount of pentose nucleic acid in the acid extract, as measured by the orcinol reaction. These observations strongly indicate that the virus preparation was free of nuclear material and that the agent is an RNA-containing particle.

With this information available, preliminary attempts were made to extract and recover an infectious nucleic acid fraction from a leukemic tissue microsome preparation containing the active virus. The phenol technique of Gierer and Schramm as modified by Colter et al. was used. The resulting aqueous extract was inoculated intracerebrally and subcutaneously into a limited number of newborn BALB/c mice. Four months after inoculation one of the animals (9 percent) which exhibited characteristic disease symptoms was sacrificed. On gross autopsy it was noted that the mesenteric lymph node was greatly enlarged, a finding highly suggestive of leukemia. Unfortunately no histological sections of this material are available. At this time (approximately 6 months after inoculation) two additional mice, still alive, show evidence of thymus involvement. These preliminary results warrant continued efforts directed toward more effective means of extracting and concentrating the nucleic acid.

2. The degree of oxidation of unsaturated lipides is measurable by the thiobarbituric acid (TBA) reaction of Kohn and Liversedge. With this reaction a study was made of the effects of added lipide oxidation products on the neoplastic (leukemic) cells in the ascites form. (See Biological No. 1 of this report.) It was found that the tumor producing activity of the ascites cells, which contain the active virus, was completely inhibited by the oxidation products of linolenic acid. The degree of inactivation of saline-cell suspensions was directly related to the concentration of added oxidation productions (lipide peroxides), as measured by the TBA reaction.

The role of lipides in the metabolism of the virus and/or tumor cell is under investigation. It is believed that these findings will contribute to a further understanding of factors concerned with the lability of the tumor virus and the role of lipides in the host-virus relationship.

Physical.--The ultrastructure of the virus and its association with the leukemic tissues are given in NCI-410 (a). Investigations into the stability of the virus source tissues have resolved the following: The sarcoma 37, the lymphosarcoma, and the virus-induced leukemic tissues maintain their capacity to produce tumors on implantation and their efficiency as virus source materials for extended periods of time when sealed and stored at the temperature of dry ice. In addition, studies on the stability of the virus concentrates under various physical and chemical environmental conditions was investigated. The results are summarized

below:

Treatment	Time of Treatment	Results
-70°C	6 months	Stable
Lyophilization	1 month	Stable
37°C	90 min.	Stable
0-8°C	24 hrs.	Stable
56°C	30 min.	Inactivated

Proposed course of project:

In addition to the studies proposed above, the following courses of investigation will be undertaken:

1. Continued biochemical investigations concerned with a more complete understanding of the nature of the virus, with emphasis on the role of lipides and nucleic acids in the metabolism of the agent and in the host-virus relationship.

2. Attempts will be made to decrease the latent period of the virus in adult animals, thus permitting a more rapid assay of virus materials. In this respect the effect of total body x-irradiation and/or urethane treatment will be examined.

3. Host studies will be continued in an effort to induce leukemia in other species as hamsters, guinea pigs, and rabbits.

4. Purification studies through the application of improved physical and cytochemical techniques.

5. The development of more refined quantitative procedures for the bioassay of the virus.

6. Quantitative time-dose-recovery studies of virus with emphasis on the relationship of concentration of virus in the leukemic tissue as seen in the electron microscope, the recovery of virus from these tissues and the onset of the disease as determined histologically.

Part B included Yes X No

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Moloney, J. B.: Biological studies on a lymphoid leukemia virus extracted from Sarcoma 37. I. Origin and Introductory investigations. J. Nat. Cancer Inst., 24: 933-951, 1960.

Moloney, J. B.: Properties of a leukemia virus. J. Nat. Cancer Inst., Monograph 4: 7-37, 1960.

Serial No. NCI-410 (a)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Maryland

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Part A

Project Title: I. Collaborative biological, biochemical and physical studies on a lymphoid leukemia virus.

II. Collaborative efforts directed toward the demonstration of viruses of possible etiological significance to other mammalian tumors.

Principal Investigator: J. B. Moloney in collaboration with those listed below.

Other Investigators:
T. B. Dunn, Laboratory of Pathology, NCI
A. Goldin, Laboratory of Chemical Pharmacology, NCI
M. Rechcigl, Laboratory of Biochemistry, NCI
A. J. Dalton, Laboratory of Biology, NCI-406
H. Kahler, Laboratory of Physiology, NCI
F. J. Rauscher, Laboratory of Biology, NCI-411
M. Fink, Laboratory of Biology, NCI-415
N. Ida, Department of Pediatrics, M. D. Anderson Hospital and Tumor Institute, Houston, Texas
R. E. Greenfield, Laboratory of Biochemistry, NCI
W. E. Heston, Laboratory of Biology, NCI
E. J. Breyere, Laboratory of Biology, NCI-418

Man Years (calendar year 1960)

Total:	2/3
Professional:	1/3
Other:	1/3

Project Description I:

Objectives.--To study through the collaboration of other investigators the biological, biochemical and physical nature of a leukemia virus and its relation to the neoplastic process.

Methods employed and major findings:

1. The pathogenesis of the virus induced leukemia in mice has been the subject of a study by Dr. Thelma Dunn of the Laboratory of

Pathology (see Part B of this report).

Male BALB/c mice were inoculated with a relatively low concentration of the agent derived from virus induced leukemic spleens. The tissues from these animals were later compared with those of mice which had received a cell free extract of normal spleen tissue. Normal or untreated BALB/c mice served as controls. Mice from each of the three groups were killed and autopsied at predetermined intervals of time following treatment. The most significant alteration and that which was first recognized in the virus treated mice appeared in the thymus. It was in this organ that leukemia first appeared. Prior to the development of this lesion a marked hyperplasia of the spleen and a localized reticulum cell hyperplasia of the lymph nodes was noted. Whole-cell transplants from the leukemic animals resulted in lymphoid neoplasms of different biological characteristics as to growth rate, and organ involvement. Transplants of the hyperplastic spleens from mice with no evidence of a true leukemia reproduced the same sequence of lesions observed after inoculation of the virus material, indicating the presence and multiplication of the leukemia virus in a host which showed no overt signs of lymphoid leukemia.

Collaborative studies are now in progress with Dr. Dunn in an effort to elucidate the pathogenesis of the virus-induced lymphoid leukemia in rats (see also NCI-410). Rats less than 48 hours old were inoculated with the mouse-derived virus. A second group of rats of the same age were untreated and considered control animals. All animals were sacrificed and autopsied when the first members of the virus-treated group showed gross evidence of leukemia. In considering the variation of host response to a given inoculum, such autopsy specimens serve as excellent material for the study of the pathogenesis of the disease in this species. The histological preparations are now under study.

2. As mentioned in a previous report, the use of this virus for the production of indicator tumors in studies of the chemotherapy of leukemia in mice has two advantages: (a) the induced neoplasm is a host tumor and (b) the induced disease is similar to that which occurs spontaneously in nature. In collaboration with Drs. A. Goldin and M. Chirigos of the Laboratory of Chemical Pharmacology selected drugs have been tested as antileukemia agents in this virus-host system. In addition, drugs of known antitumor activity were tested in a system established by the passage of neoplastic cells originally induced by the leukemia virus. Approximately three thousand animals with the virus- or whole cell-induced leukemia have been employed in these studies. The findings to date may be briefly summarized as follows: Drugs shown to be significantly effective in extending the latent period (time to death with leukemia) following inoculation of neoplastic cells are: Cytoxan, Diethylmelamine and phenylalanine mustard (Sarcolysine). Drugs of intermediate effectiveness in this cell system are: Amethopterin, dichloro-amethopterin, 6-mercaptopurine, Meticortilone and 5-fluorouracil.

Early efforts to work with virus-induced indicator tumors were somewhat difficult due to the low concentration of virus employed and the broad host variation. However, some information has been obtained on the effectiveness of three drugs, namely: Cytoxan, Methotrexate and methylglyoxal bis-guanyl hydrazine. These drugs have been found to be moderately effective in extending the time-to-death with leukemia of the test animals. Of particular interest is the marked reduction in spleen and thymus weight of those animals treated with methylglyoxal bis-guanyl hydrazine.

Chemotherapy studies on the virus-tumor system are continuing under more controlled conditions, with the development of more active virus concentrates and the use of hybrid animals which can more readily withstand drug treatment.

3. Collaborative biochemical investigations with M. Rechcigl of the Laboratory of Biochemistry are now in progress. These studies are principally concerned with the finding that the liver catalase activity is significantly depressed in animals in the terminal stage of the virus induced leukemia. Three hundred adult BALB/c mice have been employed in this study; a spleen derived virus treated group, a normal spleen extract treated group and a normal untreated group. Selected animals in various stages of the disease process were sacrificed at intervals of time following inoculation. Liver samples were stored as virus source materials, liver catalase, hemoglobin concentration and hematocrits were determined and spleen weights recorded. In addition tissue samples were taken for histological study. To date the findings are: liver catalase activity of the virus treated mice is directly related to (a) time to tumor development, (b) spleen weight, (c) hemoglobin content, and (d) hematocrit. Similar correlations were not evident in the control animals. Further studies are in progress to determine the relationship (if any) between catalase activity and (a) virus recovery and (b) the appearance of the disease as determined grossly and histologically.

4. Dr. A. J. Dalton of the Laboratory of Biology is carrying out extensive electron microscope studies on the ultrastructure of the leukemia virus and its relation to the neoplastic tissues. The following tissues from leukemic mice have been studied: spleen, thymus, lymph node, plasma, platelets, and bone marrow. In addition, studies have been made on the Sarcoma 37, the localized leukemias (lymphosarcomas) and the ascites form of these lymphosarcomas. The highest concentration of particles was found in the extracellular position in lymph nodes and thymus glands. These extracellular particles appeared poorly preserved. The intracellular particles were located in the cytoplasm within vacuoles which possessed discrete membranes. Only rarely were particles found free in the cytoplasm. Using improved staining procedures the virus particle has now been characterized as being 100 millimicrons in diameter. The particle is bound by a double limiting membrane, the inner having a diameter of approximately 85 millimicrons. The particles possess a central nucleoid which is somewhat electron dense and measures 65 millimicrons in diameter.

Examination of leukemic rat tissues (see NCI-410); spleen, thymus and bone marrow have revealed particles similar in morphology to that in the mouse leukemic tissues. Of particular and unusual interest is the finding that in the leukemic rat bone marrow sections, the virus was concentrated and localized in vacuoles within the cytoplasm of the megakaryocytes, indicating a possible site of viral multiplication.

Continued collaborative efforts will be directed toward a more complete understanding of the relationship of the virus to the disease process. Thus, quantitative studies are in progress relating particle number in the leukemic tissues and in prepared virus concentrates to tumor producing activity, histological findings, and virus recovery.

5. In an attempt to determine the density of the leukemia virus, physical and biological studies were carried out with Dr. H. Kahler and B. J. Lloyd of the Laboratory of Physiology. Virus concentrates derived from leukemic spleen tissues were fractionated in a cesium chloride solution which produces a density gradient when subjected to relatively high centrifugal force. Aliquots of the fractionated materials were assayed for virus activity, shadow cast and studied in the electron microscope. The highest concentration of particles, of the size range described above (see section 4), was found at the level where the density was calculated to be 1.24. Biological experiments designed to determine the concentration of active virus in the various density levels are in progress but definitive results are not available at this time.

6. Dr. F. J. Rauscher of the Laboratory of Biology has employed standard techniques for the growth of viruses or the chorioallantoic membrane of the chick egg, in an effort to maintain and to demonstrate the presence of the leukemia virus. A standard leukemia virus preparation was inoculated on the CAM and carried in "blind" passage for several generations. After several such passages multiple petechiae and/or lesions similar to tumor growth appeared on the membranes. Dr. A. J. Dalton has examined thin sections of these lesions with the electron microscope. Particles have been found which have the morphological characteristics of the leukemia virus as described in section 4. Representative passage materials have been extracted and the concentrates assayed in mice in an effort to determine the presence and/or concentration of the active leukemia inducing agent in the lesions. These studies are now in progress. The development of such an egg-assay system would be a highly significant contribution to the rapid development of this leukemia virus program. Quantitative biological assays could be carried out in nine days as opposed to a period of months now required for the completion of an assay experiment in mice.

7. In collaboration with Dr. M. Fink of the Laboratory of Biology, the immunological properties of the leukemia virus are being investigated (see also NCI-410). In a joint study the following homologous and

heterologous sera have been produced and tested for leukemia virus neutralization activity: normal rabbit and normal mouse, rabbit anti-normal spleen, mouse anti-normal spleen, rabbit anti-virus (purified), and mouse anti-virus (formalized). In these investigations only the mouse anti-formalized virus serum has proved to be effective in almost completely neutralizing the leukemogenic activity of the virus. Thus a final dilution of 1:10 of this antisera neutralized a $10^{-2.3}$ dilution of virus to the degree where only one of 18 inoculated animals developed leukemia within a nine months period.

Aside from the production of this homologous antiserum (see NCI-410 for production of heterologous sera) the virus has proved to be poorly antigenic. No conclusive hemagglutination or complement fixative reactions have been found to be specifically associated with the agent.

8. The mode of transmission of the leukemia virus in nature has been the subject of interest in a collaborative study with Dr. N. Ida of the M. D. Anderson Hospital in Texas. It had been observed in this laboratory that the young born to leukemic parents developed "spontaneous" lymphoid leukemias at a high incidence level after a rather extended period of time. It should also be noted that in the literature emphasis has been placed on the "egg-borne" transmission of other leukemia viruses. Controlled experiments were therefore undertaken to determine the mode or modes of transmission of this leukemia agent to the offspring. BALB/c and A/Ln mice known to be pregnant were inoculated intravenously with the leukemia virus at various stages of gestation. The mice were allowed to go to term and the offspring were subsequently foster nursed with normal untreated mothers. The results to date show that when the virus is administered during the early periods of gestation (1-4 days) a high rate of abortions or resorption of the fetuses occurs. A significantly high incidence of leukemia was noted among the young born of the group of mice which had received the virus after tne placenta was formed. The leukemia incidence varied from twenty percent o fifty percent within individual litters of the same experiment. The average latent periods (time-to-death with leukemia) were extended in time, i. e. 31 to 52 weeks. It is apparent, therefore, that at least one mode of vertical transmission of this leukemia agent is through the placental barrier. Investigations of the other possible routes (ova and sperm) of natural infections are in progress.

Project Title: Part II. Collaborative efforts directed toward demonstration of viruses of possible etiological significance to other mammalian tumors.

1. In joint studies with Dr. A. E. Greenfield of the Laboratory of Biochemistry an attempt was made to demonstrate a causative agent associated with the Dunning rat lymphosarcoma. Cell-free extracts of the tumor, harvested from Irish rats, were prepared by filtration in combination

with differential centrifugation and by differential centrifugation alone. The concentrates were inoculated into newborn rats of the same species. To date, 10-12 months after inoculation of the animals, no lesions have appeared in the hosts which can be directly attributed to a specific virus.

2. A series of eight tumors (carcinomas and sarcomas) which have been maintained for periods up to four years in various strains of mice in the laboratory of Dr. W. E. Heston were "screened" for the presence of etiologic agents. Cell-free extracts of the tumors were prepared by centrifugation and by filtration in combination with differential centrifugation. The concentrates were inoculated into compatible newborn animals. Thus far only negative results have been obtained with each of the tumors employed as source material. This work is being done in collaboration with Dr. Heston and George Vlahakis of the Laboratory of Biology.

3. Three previous attempts to demonstrate a causative agent in a plasma cell tumor (70429) maintained in C3H mice by Dr. M. Barrett have proved negative. Recently Dr. E. J. Breyere of that Laboratory has shown that parity influences host susceptibility to "foreign" tumor implants. Employing this phenomenon Dr. Breyere has been able to transplant and maintain the C3H plasma cell tumor, 70429, in BALB/c mice. In collaboration with Breyere an effort was made to recover an etiologic agent from the tumor growing in BALB/c mice, assuming that this strain might serve as a more favorable medium for the growth and multiplication of viruses. A cell-free extract was prepared by differential centrifugation and the resulting suspension inoculated into newborn BALB/c mice and compatible F_1 hybrids. One percent of the animals developed a generalized neoplasm, a tumor which, through immunogenetic studies, was found to be a BALB/c tumor. These tissues were harvested and transplanted into adult animals. The biological behavior of the transplanted tumor in BALB/c mice was similar in all respects to the original neoplasm grown in the C3H strain. A specific characteristic was the high degree of ovarian infiltration. Studies with cell-free extracts of the transplanted tumor (originally "virus induced") are now in progress in an effort to concentrate the agent in the source tissues and thus enable a more complete identification of the agent.

Part B included Yes X No

PHS-NIH
Individual Project Report
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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Dunn, T. B., Moloney, J. B., Green, A. W. and Arnold, B.: Pathogenesis of a virus induced leukemia in mice. J. Nat. Cancer Inst. 26: No. 1, 1961.

Honors and Awards relating to this project: None

Serial No. 411 (a)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Biological studies of two murine leukemia viruses in mice and embryonated hens eggs.

Principal Investigator: Frank J. Rauscher

Other Investigators: John B. Moloney, NCI-410
A. J. Dalton, NCI-406

Cooperating Units: None

Man Years (calendar year 1960):

Total	1
Professional:	1/3
Other:	2/3

Project Description:

Objectives.--To devise methods of measuring viral potency by studying characteristics of the virus other than frank infectivity in its natural host, and to compare the responses of various host systems to 2 viruses which produce the same general type of disease but which apparently differ in their pathogenesis.

General Statement.--In 1957 Schoolman and Schwartz of the Hektoen Institute, Cook County Hospital, Chicago, Illinois, reported studies on a leukemia virus of mice. The virus was reported to produce a generalized lymphoblastic disease in non-inbred, adult Swiss mice to an incidence of 60 to 100 percent in 2 to 6 weeks. Of major interest was the fact that although the disease in mice was characterized by lymphadenopathy, moderate spleno-hepatomegaly and a pathognomonic mesenteric tumor the causal virus could be isolated only from the brain, and in no instance from any other tissue. Numerous investigators in different laboratories have tried to repeat this work and almost invariably have failed. Schwartz and associates have also reported difficulty in repeating some of their original observations.

Studies of this investigator (FJR), at Rutgers University and during the past 7 months at the National Cancer Institute have shown that the disease is caused by a virus but that the expression of the

disease in mice apparently is influenced by as yet unresolved factors inherent in the virus and in the host response to the virus.

Methods Employed.--Techniques for enhancing the potency of tissue extracts and of enhancing host response to the virus which have been used successfully for other host-virus systems, are being employed. Attempts are also being made to cross species lines by inoculating the virus into embryonated hen and Japanese quail eggs by various routes and under various conditions. Potential manifestations of viral activity other than frank infectivity are being explored, such as hemagglutinating, antigenic, toxic, and interfering activities of the virus. The pehnomenon of viral interference is being studied by inoculating mice or embryonated hens eggs with inocula known to contain or presumed to contain the leukemogenic virus followed at various time intervals by a second inoculation of one of 7 avian, human or mouse viruses. A qualitative or quantitative decrease in the response of the host to the challenge virus under properly controlled conditions may be expressed as interference of the challenge virus by an agent present in extracts of leukemic tissue.

Major Findings:

A. Schoolman-Schwartz Mouse Leukemia Virus (SSLV).--Attempts have been made to increase the potencies of tissue extracts so that virus preparations of high infectivity and stability may be obtained for quantitating this system. The potency of the best virus preparation so far attained has been a leukemic incidence to palpation or death of approximately 30-35 percent occurring between 1.5 to 2.5 months after inoculation. Repeated attempts to isolate infective virus from tissues other than brain were unsuccessful. Extracts of tumor tissue were found to contain substances capable of completely inhibiting the most potent preparations of SSLV available. Experiments in progress indicate that the inhibitor is composed of a soluble and a particulate component.

Studies designed to infect or to induce some measurable response in animals other than the mouse are currently in progress. The virus has been serially passaged on the chorio-allantoic membrane of embryonated hens eggs. No discrete lesions were observed grossly or histologically; however, when eggs were inoculated with CAM extracts of the first and sixth serial passages and then challenged with influenza virus, a 90 to 99 percent reduction in the titer of the challenge virus was noted. The same CAM extracts when heated to 60°C/30 min., or extracts of CAM's from eggs inoculated with normal mouse tissue failed to depress the titer of influenza virus. Extracts of CAM's showing this interfering activity have not produced leukemia in mice, as of approximately 2 months post inoculation.

B. Moloney Mouse Leukemia Virus (MMLV).--In collaboration with Dr. J. B. Moloney as co-principal investigator, studies in eggs similar

to those described above are being done with MMLV. To date the virus has been through 12 serial CAM and 4 serial intravenous passages in embryonated hens eggs. Japanese quail embryos have also been used with no positive results to date. In hens eggs, discrete areas of CAM involvement were seen on the fifth, sixth and ninth passages. The appropriate control eggs remained negative. These studies have begun only recently and it, therefore, would be hazardous to attach too much significance to a lesion which has not been properly characterized as to a true cause and effect relationship to the virus. Dr. A. J. Dalton has seen virus-like particles in thin sections of CAM lesions of the sixth serial CAM passage. The significance and identity of these particles remains to be determined. Extracts of CAM tissue from several serial passages have been inoculated into newborn BALB/c/An mice. This experiment has not progressed an adequate amount of time to be reported here. (See NCI-410 by Moloney.)

Significance of Bio-Medical Research:

Several murine tumor viruses have been isolated in recent years. Knowledge of their differences and similarities is sparse, primarily because each virus has had to be studied by and of itself. Among other things, all of these agents are characterized by relatively long latent periods. The studies described in this report may facilitate more rapid methods of assay and comparison of at least 2 of these viruses in host-systems in which contagious spread or cross contamination of virus strains is minimized.

The SSLV induced leukemia appears to offer an excellent model in which neoplasia is known to be produced by a virus but in which the disease or recovery of the virus from the disease can not be predictably or consistently manifested. It is conceivable that an understanding of factors seemingly inherent in both virus and host which enhance or depress the production of leukemia in the mouse will help determine the manner in which human and other mammalian neoplasias of unknown etiology may be extracted and inoculated into more receptive indicator hosts (see NCI-420 by Bryan).

Proposed Course of Project:

Methods which have been used successfully for studying the necrotizing or non-tumorigenic viruses will continue to be used in attempting to develop less time consuming quantitative methods of assay. Properties of mouse leukemia viruses other than infectivity will be explored. The apparent ability of at least one mouse tumor virus (SSLV) to interfere with influenza virus will be exploited. It is planned to test leukemic tissue extracts from which virus can or can not be isolated against several human viruses (mumps, influenza A and B); and against the viruses of Newcastle disease, infectious bronchitis, laryngo-tracheitis, fowl pox and Rous sarcoma.

Emphasis will be placed on increasing the potency of SSLV preparations so that enough virus is available for quantitative studies. To this end, host factors such as soluble inhibitor and antibody, and the role of the natural or acquired viral flora of the host on the manifestation of induced neoplasia will be examined.

Part B included Yes No X

Serial No.NCI-411 (b)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Characterization of Japanese Quail (Coturnix coturnix japonica, Temminck and Schlegel) as a new host for virus studies with emphasis on determining their potential usefulness in cancer research.

Principal Investigator: Frank J. Rauscher

Other Investigators: Dr. James A. Reyniers and associates.
John P. Kvedar.

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To evaluate the use of Japanese quail embryonated eggs and post-hatched chicks in virus and cancer research. To determine the relative susceptibilities of this host to the necrotizing and tumorigenic viruses known to be infectious for embryonated hens' eggs. To determine whether or not this animal will support the measurable growth of viruses which grow poorly or not at all in other host systems.

General Comments: Japanese quail embryos or post-hatched chicks offer several desirable advantages over their fowl counterparts. Adult quail weigh on the average 90-120 gms.; they become sexually mature in approximately 14 weeks whereupon the hens lay 5 to 6 eggs per week. The embryos are hardy and hatch after an incubation period of 14-15 days. Adequate diets are available as is all equipment necessary for their maintenance. The small size, economy of maintenance, rapid generation time, and relative resistance to changes in incubating temperatures are advantageous to studies involving genetics of the host itself or of viruses inoculated into the host maintained under different environmental conditions. Experiments which require holding animals for long periods of time would require much less space than would the use of chickens.

Methods Employed: No new methods of particular scientific interest have been used in this project. Techniques of handling, of preparing eggs for inoculation, and of inoculating eggs are well known and in general use. However, the modification and adaptation of standard hen's eggs techniques to quail eggs was of primary importance to this project. A brief summary of methodological findings is presented below.

Major Findings:

Methodological: The 6-7 day old embryo was found to be optimal for allantoic sac inoculation. Yields of allantoic fluid amounted to 1 to 3 ml, 2 to 4 days after inoculation. 0.05 to 0.2 ml can be inoculated by this route with no apparent injury to the embryo. Quail eggs were also inoculated intravenously, amniotically and into the yolk sac. Of major interest to virus studies is the fact that the chorio-allantoic membrane (CAM) of 6-8 day eggs may be dropped in approximately 80-90% of eggs. It was found that the dense brown-black shell pigment was easily removed from freshly laid eggs by gentle buffing with a steel soap pad. The resulting white shell allowed greater visibility of the embryo and of its membranes during candling. Histologically, tissues of the embryo and of its extra-embryonic membranes were found to be similar to those of the hens' egg. The CAM of the quail egg was found to be moderately more variable in its over-all thickness and in the number of cells comprising the chorionic and allantoic layers of the CAM.

Experimental: The response of Japanese quail eggs to the viruses of Influenza A, Influenza B, visceral lymphomatosis, fowl pox, Rous sarcoma, infectious laryngo-tracheitis, Newcastle disease, and infectious avian bronchitis, and the nature of these viruses as recovered from quail tissues has been studied. With the exceptions of Rous sarcoma virus (RSV) and visceral lymphomatosis virus (VLV) quail eggs supported the growth of and manifested the same general pathology as viral infection of embryonated hens' eggs. The dilution endpoint of virus inoculated into quail eggs and the amount of virus recoverable from infected quail was almost identical to that following

infection of hens' eggs. The various parameters of infection have been determined by quantitative studies involving back infectivity titrations in hens' eggs of quail-adapted virus. Also, hemagglutinating activities of virus for quail and chicken erythrocytes , neuro and pneumo-toxic effects of quail vs. chick strains of virus for mice have been tested and were found to be comparable. In summary, the viruses of Influenza A and B, Newcastle disease, fowl pox, infectious avian bronchitis and laryngo-tracheitis as recovered from infected quail eggs of from 1 to 15 serial passages showed the same characteristics as virus grown in embryonated hens' eggs. However, attempts to show differences in viral progenies and in host responses to these viruses have not been exhausted.

Inoculation of RSV onto the CAM results in the production of tumor foci. However, the RSV-quail system appears to differ substantially from that of embryonated hens' eggs in several respects. The most important of these are: (1) Failure to support serial passage of the virus. Seven attempts to passage RSV for more than 3 serial passages on the CAM of quail eggs were unsuccessful. This was true regardless of the amount or source of virus used to initiate each passage. (2) The production in eggs which failed to develop gross lesions of a substance inhibitory to RSV. When extracts of CAM's from inoculated but negative quail eggs were mixed with or inoculated prior to or shortly after infectious RSV, the titer of the latter was suppressed by 1 to 4 logs. The possible relationship of this phenomenon to "interferon", to the resistance-inducing-factor (RIF) of Rubin and to lymphomatosis virus is being studied. (3) Susceptibility of adult quail significantly greater than that of their embryonated eggs. Preliminary studies indicate that adult quail will support serial passage of RSV. The dilution endpoint assay for tumor production and the amount of virus recoverable from quail tumors was found to be similar to that in chickens. (4) The number of pocks produced on CAM's and the amount of virus extractable from CAM's is apparently unrelated to the amount of virus contained in the infecting dose.

Significance to Bio-medical Research: The finding that Japanese quail may be manipulated in a manner similar to that of embryonated hens' eggs, and, that they are susceptible to at least 6 different viruses indicated that this host may be suitable for virus studies. Their small size before and after hatching, their ease of maintenance, and their rapid generation time makes them more suitable as an avian host in studies requiring long periods of observation. If their susceptibility to the tumor viruses of the avian complex can be demonstrated, they will be extremely useful in basic studies of these agents. It is possible that this host may be adaptable to the isolation of agents from some human tumors.

Proposed course of project: Embryonated quail eggs will be tested for their susceptibilities to other viruses representative of both human and animal diseases. Viruses which grow poorly or not at all in other host systems will be tested in quail eggs. The pathogenesis of RSV infected embryos and of post-hatched chicks will be studied along the following lines:

(a) the apparent unique difference in the susceptibility of the post-hatched quail over its embryo.

(b) the nature of the substance inhibitory to RSV occurring in quail embryos of the 2nd to 4th serial passages of RSV.

(c) the biology of the Japanese quail; its normal viral flora and its potential usefulness as a new host for virus-cancer studies.

(d) the comparative susceptibility of quail-embryo tissue cultures to RSV and to other viruses.

(e) Dr. Robert F. Zeigel is preparing to study the ultrastructure of normal and infected quail tissues with the aid of an electron microscope.

Part B Included Yes No X

Serial No.NCI-411 (c)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The effect of various biological, chemical, and physical agents on viral potency, and on host response to Rous sarcoma virus (RSV).

Principal Investigator: Frank J. Rauscher

Other Investigators: Mary A. Fink - NCI-415
John P. Kvedar

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: The objectives are: (A) To determine whether infective virus can be recovered from non-infective complexes with immune or inhibitor substances, and (B), to determine the effect of conditioning the host with various agents on production by the host of infective virus or of substances inhibitory to the virus.

Methods Employed: Bioassays of virus activity as well as of substances which inhibit virus activity are carried out by means of the widely used chorioallantoic membrane-pock counting technique.

Efforts to separate the virus from complexes with immune or inhibitory substances were made using the principles of differential ultracentrifugation and of fluorocarbon (Genetron 113) extraction.

Major Findings:

(A) Attempts to recover infective virus from non-infective complexes of the virus with immune or inhibitor substances.

(1) The reported ability of fluorocarbon compounds to effect a separation of certain viruses from specific antibody was tested with RSV. Fluorocarbon (Genetron 113) treatment of a non-infective mixture containing RSV plus specific antibody failed to effect a recovery of infective RSV, under the conditions of the experiment. Studies of this type with substances such as the fluorocarbons have only recently been undertaken. Evaluation of their usefulness in extracting infective agents from biological tissues or fluids must await further work.

(2) The separation of active RSV from a non-infective mixture containing specific antibody has been accomplished by ultracentrifugation. In some experiments approximately 5 - 30% of RSV was recovered from an inactive mixture following centrifugation at 30,000 to 100,000 x g for varying periods of time. The efficiency of recovery has shown wide variation and the results of some experiments have not been repeatable on a quantitative basis. Further work is needed to clarify these data.

(3) Simple dilution of immune mixtures occasionally results in a recovery of infective RSV. Preliminary data indicate that the composition of the diluent, its temperature, and the magnitude of the dilution all contribute to the amount of infective RSV recovered.

(4) Certain non-infective lesions produced by low doses of RSV or non-infective lesions produced in Japanese quail eggs (see NCI-411 (b)) have been made to yield infective virus. Trypsinized, single cells from such lesions were inoculated onto the CAM's of hens' eggs. Approximately 2 to 3 logs of RSV have been demonstrated in some extracts of tumors which subsequently developed on the CAM following this procedure.

(B) The effect of conditioning the host with Freund's adjuvant (complete) on production by the host of infective virus or of substances inhibitory to the virus. - Dr. Mary A. Fink, co-principal investigator, NCI-415.

These studies may be divided into 2 parts; (1) treatment of the host before infection with RSV, and (2) treatment of the host after infection. Each part involves the assay of several hundred serum and tumor samples from individual chickens. The results of these studies, only a small portion of which have been completed, are presented below.

(1) Pretreatment. When 2-6 week old chicks were inoculated subcutaneously with Freund's adjuvant extensive granulomatous lesions developed at the site of inoculation. The reaction appeared in 4-5 days and in some cases attained a weight of 20 - 30 gms, 25 days after inoculation. Granulomatous tissue was successfully passaged through at least 3 transplant generations in young chicks. When chicks were inoculated into the granuloma with approximately 0.01 ED 50 of RSV 15 days after receiving Freund's adjuvant, their granulomas grew at an accelerated rate and appeared to invade adjacent muscle. Extracts of these growths yielded 2 to 4 logs of RSV. The same dose of RSV (0.01 ED 50) failed to produce tumors in control birds pretreated with saline. RSV could not be demonstrated in tissue extracts from control birds. The incidence and severity of metastases were also increased in birds pretreated with adjuvant. Fifty per cent of adjuvant conditioned chicks inoculated with 10,000 ED 50 of RSV developed visceral metastases whereas 0 per cent developed metastases in chicks pretreated with saline and infected with the same dose of RSV.

(2) Post-treatment: When chicks were inoculated with a dilution of virus which failed to produce tumors and then inoculated with adjuvant, 85 per cent developed relatively large amounts of anti-RSV antibody in their sera. Antibody was not produced in control chicks which received the same dose of virus but which were not inoculated with adjuvant. The sera of similar groups of chicks inoculated with 12 different dose levels of RSV are being studied. In general, negative birds challenged with adjuvant develop antibodies to RSV, while the sera of control negative birds challenged with saline, remained free of significant levels of antibody. This relationship became less distinct at the 10^{-10} level of RSV. Coincidentally, the total particle count of the virus preparations used in this experiment was about 10^{10}/ml.

Significance to Bio-medical Research: Procedures or substances which enhance host response to a virus or which allow more potent preparations of viruses to be made would be a desirable inclusion in attempts to isolate or to demonstrate viruses from diseases of unknown etiology. In addition, the ability to produce a measurable response by minimal amounts of virus should enable one to study the initiation and course of a disease as it more naturally exists in nature.

Proposed course of project: Studies designed to combine and then to separate infective virus from a non-infective complex will be continued. In addition to those methods already being used, chromatographic cellulose columns will be employed in attempts to recover infective virus. The studies concerning the effect of pre- or post-treatment of chicks with Freund's adjuvant will be completed and extended to confirm those points discussed above.

Part B Included Yes X No

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Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Fink, M. A., and Rauscher, F. J.: A simple method for the preparation of potent chicken anti-RSV serum. J. Nat. Cancer Inst. Accepted for publication.

Honors and Awards relating to this project:

Serial No. NCI-415(a)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Application of immunological techniques to the problem of demonstrating viruses in human and other tumors, either directly or by revealing the presence of specific antibody.

Principal Investigators: Mary A. Fink

Other Investigators: None

Cooperating Units:

W. R. Bryan and associates (NCI-420)
J. B. Moloney (NCI-410)
F. J. Rauscher (NCI-411)
M. B. Melroy (NCI-419)

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

1. Objectives: The ultimate objective is to provide an immunological tool for the recognition of viruses which are associated with human tumors as etiological agents. Such a tool, once developed, can be applied to the identification of viruses with known antisera, or the identification of antibodies in the serum of tumor-bearing animals, with known viruses.

Inasmuch as animal tumor virus preparations are available, and those from the human are not, the problem has been approached using as models the Rous sarcoma virus (RSV) of chickens, and the Moloney leukemia virus (MLV) of mice.

2. Methods Employed: Preliminary work, initiated in 1959 and continued this year, yielded methods for the production of high titred, specific antiserums against these two viruses, as measured by neutralization tests.

Both heat-killed and formalin-killed viral preparations of RSV and formalin-killed MLV preparations were found to produce, when incorporated in Freund's complete adjuvant, a high-titred neutralizing antibody. The preparation of RSV antisera in chickens, and MLV antisera in BALB/c mice, yields a much more specific anti-viral antibody than if prepared in heterologous species, in which the major antigenic stimulus is provided by the species antigens associated with the viral vaccines. Assays of the neutralizing content of the sera were carried out by Dr. J. B. Moloney (MLV) and Dr. F. J. Rauscher (RSV).

In a highly controlled experiment, designed to keep all samples as unknowns, the following were included: serum from normal BALB/c mice; serum from BALB/c mice injected with heated or formalinized BALB/c lymphoid tissue preparations, prepared identically to the viral preparations; and serum from mice injected with either formalinized or heat inactivated lymphoid tissue preparations from leukemic mice. In addition, the serums from normal rabbits, rabbits injected with a highly purified preparation of MLV, and with a similar preparation from non-leukemic tissue, were included.

Dr. Moloney found that only the serums from BALB/c mice injected with HCHO treated leukemic tissue were able to neutralize the MLV virus. Results of this experiment are now being tabulated preparatory to publication.

The chicken antiserums vs. RSV were titrated for neutralizing activity by Dr. F. J. Rauscher, using an egg embryo-membrane technique. The results of this study, in manuscript form, have been accepted for publication by J.N.C.I.

Testing of antisera: Once antiserum preparations of high potency were available, a concerted effort has been made to find in vitro serological tests capable of assaying for the presence and the relative concentration of anti-viral antibodies.

After screening many serological tests with both RSV and MLV, the two most versatile were selected for intensive study. Technical variables have been manipulated within these two test systems - complement fixation, applied to both RSV and MLV, and tanned erythrocyte agglutination, so far applied only to RSV - in hopes of uncovering the exact set of circumstances for recognizing the antigen-antibody reaction.

a) RSV -. In all testing procedures, the antigen used was a standard virus preparation prepared in Dr. W. R. Bryan's laboratory.

1) complement-fixation.

No fixation of complement could be demonstrated using a standardized system based on the fixation of guinea pig complement (C').

On the knowledge that not all species of antibody can fix guinea pig C', the species source of C' was varied to include chicken, rabbit and human. In no case was C' fixation observed.

The precipitation of chicken antibody with its homologous soluble antigen is known to be enhanced when the salt concentration is increased to 8%. To the limits of tolerance of the test system, various salt concentrations were used in C' fixation tests; none produced positive complement fixation.

Recently a reference appeared which said that the addition of fresh normal chicken serum to the reaction mixture would supply an unknown substance destroyed on heating and dilution of chicken serum, which was necessary for complement fixation to take place. This approach is currently under study. Trials with two different batches of fresh normal chicken serum have not confirmed the other work, but it is hoped that leads will be uncovered which will help solve the problem.

A complicating factor in both the chicken and mouse serum work has been, despite careful handling, the occurrence with great frequency of anticomplementary serums. Recently we have adopted a Kaolin absorption technique which successfully rids the serum of anticomplementary activity. The concurrent effect of this treatment on viral antibody content has not yet been determined, but has been reported to be negligible in other systems.

2) Tanned erythrocyte agglutination

One of the most sensitive methods for the detection of antibody to soluble protein antigens is based on the hemagglutination of erythrocytes which have been treated with tannic acid, to which proteins will then adhere.

This test has been applied to the titration of RSV antibodies in chicken antisera. The many variables - reactivity of different lost of virus - optimal concentration of virus - optimal time and conditions of contact of virus with tanned cells - are all being investigated in an attempt to demonstrate a quantitative difference in the antibody content of chicken antiserums known to have a different content of neutralizing antibody.

This test will continue to be explored to the fullest. It is felt that if the optimal conditions are found with RSV, the possibility of application of a similar system to other tumor viruses would be good.

b) MLV -

Using the antiserum prepared in BALB/c mice with formalin treated virus in adjuvant, the complement fixation test has been thoroughly explored. There is a good indication that C' fixation does occur in low titre, but a completely satisfactory antigen treatment has not yet been found. The tanned erythrocyte technique will also be tried with MLV.

c) Polyoma virus

Various serums from mice, for both control and diagnostic purposes, have been assayed by the hemagglutination-inhibition test for polyoma virus antibodies. The testing has been done by Mr. Melroy.

Other tests

Several unsuccessful attempts have been made to demonstrate antibody to both RSV and MLV by eliciting a delayed-type hypersensitivity response to an intradermal injection of virus. We have also been unable to show an anaphylactic response in the mouse to MLV; this technique will be pursued, however, now that we know better how to immunize the animal.

3. Major findings:

A) Practical methods for the production of antisera against RSV and MLV have been found which will most likely be applicable in other tumor virus systems.

B) Leads have been obtained toward finding in vitro techniques for assaying antibody to tumor viruses.

4. Significance to bio-medical research and the program of the Institute:

For maximum progress in the tumor virus field, it is almost imperative that serological means of identification of tumor viruses and/or antibodies to them be found. All of our past experience indicates that this is a realistic goal. While the going may appear to be slow, it is absolutely necessary that all ramifications of test systems be studied in detail and be rigidly controlled, so that a truly useful and reproducible technique will be available for the identification and comparison of viruses found to be associated with malignancies in animals, and especially in man.

5. Proposed course of project:

The present effort to adapt either complement fixation or tanned erythrocyte agglutination to the tumor virus problem will be continued. The trial of every new practical technique, and attempts to innovate will be continued.

Part B Included Yes X No

Serial No. NCI-415(a)

PHS-NIH
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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Fink, M. A., and Rauscher, F. J.: A simple method for the preparation of potent chicken anti-Rous sarcoma virus serum. J.N.C.I., accepted for publication.

Honors and Awards relating to this project:

By request from Tissue Bank Department, National Naval Medical Center, appointed Lecturer in Immunology at National Naval Medical School.

Serial No. NCI-415(b)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Attempts to cross species barrier of infectivity in viruses as applied to the establishment of human tumor viruses in mice.

Principal Investigator: Mary A. Fink

Other Investigators: None

Cooperating Units: Dr. W. R. Bryan and associated (NCI-420)
Dr. F. J. Rauscher (NCI-411)

Man Years (Calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

1. Objectives: The ultimate objective is to have available a tool for demonstrating that a virus isolated from and believed to be etiologically associated with a human tumor is capable of initiating a specific disease--malignant or otherwise--in an experimental animal.

If either the virus or the host could be artificially manipulated to the extent that infection with a foreign virus could take place, at least the solution to the problem would have a beginning. For this reason, an attempt has been made to induce in mice a recognizable, reproducible change by the injection of a known, alien, tumor virus, namely the avian Rous sarcoma virus.

2. Methods employed: Various techniques have been attempted to alter the receptivity of the mouse to infection with Rous sarcoma virus (RSV).

A) Hundreds of newborn mice have been injected by one of a variety of routes--intraperitoneal, subcutaneous, intracerebral, intranasal--with RSV. All of these injections were without effect.

B) Out of curiosity, and in an attempt to alter the resistance mechanisms of the host via insult, the RSV was emulsified with Freund's adjuvant, and has been injected into a number of newborn mice. This treatment has been found to produce irregularly in the mouse a lethal syndrome characterized grossly, after either intraperitoneal or subcutaneous injection, by the appearance of a hemorrhagic, gelatinous exudate in the abdominal cavity. It has not been possible to recover RSV from this material by injections into either the chicken wingweb or the chorioallantoic membrane of the chick embryo.

In the course of the work, however, it was determined that the injection of complete Freund's adjuvant, but of none of the components alone, or the adjuvant without tubercle bacilli, caused the production in young chickens of a massive, rapidly growing granulomatous reaction. Following these observations, various projects have been undertaken to ascertain the effect of adjuvant on RSV infection in the chicken, especially in regard to whether or not viral potency is enhanced, or antibody production altered. These projects, done with Dr. F. J. Rauscher as co-principal investigator, are discussed more extensively in his report.

C) The technique of injection of mouse embryos in utero has been practiced, and a limited number of trials made with RSV. Unfortunately, cannibalization of the babies, even after 48 hours of life, has prevented the realization of any results.

D) Emphasis was placed this year on attempting to determine the relative efficiency in preventing an antibody response in later life, of injecting the neonatal mouse with chicken tissue, with cortisone, or with both.

An experiment was designed in which the microsome fraction of normal chicken liver was injected by various routes on the morning of birth, into one group; control mice received nothing.

At two months of age, each group was divided, and ½ given cortisone, the other ½ not. Then all of the animals were injected with chicken liver microsomes in Freund's adjuvant, bled after 3 weeks, and the serum individually titrated by a tanned-erythrocyte agglutination technique for antibody reactive with chicken liver microsomes. Results of this experiment showed a negligible effect on depression of antibody formation by the neonatal injection; but a definite depressive effect with either cortisone alone, or cortisone plus the neonatal injections.

The next experiment was designed to determine the daily amount of cortisone necessary to suppress completely an antibody response to the chicken antigen. This dose was determined, and was found to be compatible with life over the three-week injection period.

Groups of mice 3, 4, 5, and 6 weeks old were then injected by various routes with RSV 48 hours after beginning a three-week course of cortisone. Many of the experimental mice died, grossly exhibiting symptoms of a hemorrhagic intestinal syndrome highly reminiscent of that seen in the Freund's-RSV mice. Beginning several days later, an approximately equal percentage of the control animals also died, with autopsy findings grossly similar to the experimental animals. It is now believed that the RSV may have had an effect, as manifested by earlier death, but that the dosage of cortisone was excessive.

This type of experiment will be repeated as time and animal space permit.

Throughout this work various materials from RSV injected mice have been transferred from mouse to mouse, never with definitive findings. Many of these mice are currently under observation.

Major findings:

A) The only concrete finding is that cortisone alone can completely suppress the formation of antibody to chicken protein in mice; but neonatal injection of antigen cannot.

B) A strong suspicion is held that the RSV is adversely affecting the mouse, although the results are too irregular for any judgment to be made at this time.

4. Significance to bio-medical research and the program of the Institute:

If a technique can be found for breaking down the species barriers to infection with tumor viruses, it would conceivably be possible one day to fulfill Koch's postulates in establishing viruses as the true etiological agents of some human malignancies. With this possibility in mind, no matter how remote, no amount of effort can be spared, and no approach left unexplored, in attempting to find a solution to this difficult problem.

5. Proposed course of project: Continuation of studies with RSV, repeating the in utero and cortisone experiments, and investigation of the effect of X-ray on antibody production and the growth of RSV in the mouse; application of any promising technique to the human tumor program.

Part B Included Yes No X

Serial No. NCI-415(c)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Investigation of antigenic changes of tissue culture cells subjected to various growth environments, as compared to cells derived from fresh tissue.

Principal Investigator: Mary A. Fink

Other Investigators: None

Cooperating Units: Dr. Virginia J. Evans (NCI-428)

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

1. Objectives: (A) To determine whether or not there are antigenic alterations in cells which have undergone cultivation in tissue culture; and (B) to seek and to confirm methods for species identification of tissue cultured cells.

2. Methods employed: Cells: strains of tissue cultured monkey kidney and human epithelium were prepared by Dr. V. J. Evans, thoroughly washed, and frozen at -20° until used. Fresh monkey kidney cells were prepared by Dr. Evans by a standard trypsinization technique, washed and stored at -20° until used. Fresh human epithelium was obtained through the courtesy of the Tissue Bank of the National Naval Medical Center; cells obtained from this tissue were stored as before.

Because one objective of the experiment was to test the species derivation of the cells by a hemagglutination technique described by Brand, his protocol for immunization was followed. In brief, guinea pigs were injected with standardized preparations of the cells in Freund's adjuvant. After an interval of time, they were bled and their serums tested for antibodies capable of agglutinating erythrocytes of human, monkey, rat, rabbit, chicken and mouse.

Pigs in each group were then equally divided, one-half being tested for the development of delayed-type hypersensitivity by the intradermal injection of standardized cell-free protein extracts derived from the homologous fresh and tissue cultured cells, and of serum from the same species. Uteri from the remaining animals were tested by the Schultz-Dale technique for reactivity with the various preparations.

3. Major findings: A) The red cell agglutination technique revealed that the monkey and human tissue cultured cells stimulated the production of antibodies which were specifically reactive with only homologous erythrocytes, thus confirming the applicability of the Brand test, and giving assurance that these two cell lines were indeed as labelled. Very surprisingly, neither the fresh monkey nor fresh human cells incited the production of antibody reactive with homologous erythrocytes.

All sera were also tested by the Ouchterlony double diffusion technique.

B) The tests for delayed-type hypersensitivity revealed that both the tissue cultured and fresh tissues contained common antigens. In a comparison of fresh vs. cultured human epithelium, it was noted that pigs injected with fresh tissue reacted to human serum, while those injected with cultured tissue did not.

C) In tests for immediate hypersensitivity (Schultz-Dale) there was again evident a marked diminution or absence of reactivity in the uteri from pigs injected with tissue cultured cells, but not in those from pigs injected with fresh tissue, when in contact with homologous serum.

These results, if reproducible, strongly suggest an antigenic alteration of cells in tissue culture. Based on research to date, the process might be visualized as the loss of a surface antigen, thus uncovering or increasing the availability or potency of the antigen responsible for hemagglutinating antibodies. On the basis of the hypersensitivity reactions, a loss of the antigen responsible for antibodies against antigens also contained in the serum is a possibility.

4. Significance to bio-medical research and the program of the Institute:

The need for assessing the antigenic constitution of cells during tissue culture has long been apparent. Practically, a technique for determining the species of origin is a necessity in laboratories where one error in labelling, or in the mixing of cultures can lead directly to cluttering the literature with erroneous findings on such things as metabolic behavior of cells, on the virus spectrum of cells, and on the nutritional requirements of cells. More basically, the determination of antigenic changes in cultured cells could conceivably reveal information pertinent to explaining a concurrent progression to the malignant state; and might also yield information useful in problems of tissue banking for clinical usage. If histocompatibility antigens are affected, the possibility exists that tissue cultured cells containing tumor viruses from one species might be more readily transferrable to experimental animals of a different species.

5. Proposed course of project: Currently an attempt is being made to repeat part of this work on a small scale, using fresh and tissue cultured cells derived from the mouse.

Being limited by lack of personnel, animal space and time, we can only hope to pursue this problem on a small scale, even though it appears to be one which would yield to intensive investigation, and would be exceptionally valuable in the fields of virology and cancer.

Part B Included Yes No X

Serial No. NCI-416
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Viral Therapy of Tumors. I. Adaptive Measures for Increased Oncolysis in Cases of Advanced Carcinoma of the Cervix.

Principal Investigator: Robert A. Manaker

Other Investigators: Edward D. McLaughlin, Robert B. Couch

Cooperating Units: This project complements the clinical aspects of viral therapy of carcinoma of the cervix conducted under the direction of Dr. Robert R. Smith, Chief, Surgery, NCI, and extends the work initiated by Drs. Robert J. Huebner and Wallace P. Rowe, Laboratory of Infectious Diseases, NIAID, 9 - 752 - C.

Man Years (calendar year 1960):
Total: 1/3
Professional:
Other: 1/3

Project Description:

Objectives: To continue the laboratory aspects of an investigation to determine the extent to which the oncolytic properties of Coxsackie B-3 virus could be enhanced by tumor-to-tumor passage.

Methods: Tumor-to-tumor passage of virus was conducted in patients with carcinoma of the cervix by Dr. Edward McLaughlin under the direction of Dr. Robert R. Smith. The viral inocula supplied for the clinical investigation were prepared in HeLa cells and represented the 10th and 11th tumor passages of Coxsackie B-3 virus. Four patients with negative or low neutralizing antibody titers to the virus were inoculated.

Major Findings: Two patients with no neutralizing antibody were inoculated with 10th passage virus. Virus was recovered from cervical swabs taken through the 3rd and 4th days post-infection, but not thereafter. Antibody titers rose to 512 to 1024 within 2 weeks. The 3rd patient inoculated had a pre-virus antibody titer equal to 4. No virus was recovered after the first day. Rise in antibody titer was evident on the 6th day post virus. No cervical swabs were available for the last patient prior to the 7th day post injection. No virus was isolated. Increase in antibody was evident in serum drawn on the 8th day. There was no evidence of significant increase in oncolysis due to virus adaptation. Clinical findings will be reported by Dr. Robert R. Smith.

Significance: The limited usefulness of tumor-to-tumor passage of Coxackie B-3 virus to achieve increased oncolysis of tumors of the human uterine cervix is indicated.

Proposed Course: Cooperation in the clinical investigation of viral therapy of human tumors will be continued.

Part B Included: Yes No X

Serial No. NCI-416(b)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The Application of Tissue Culture to the Study of Tumor Viruses. II. Viruses Associated with Human Tumors.

Principal Investigator: Robert A. Manaker

Other Investigators: W. Ray Bryan (Director of group program), M. A. Fink, J. B. Moloney, F. J. Rauscher, Dorothy Calnan, J. P. Kvedar

Cooperating Units: H. B. Andervont and associates, NCI-400.
A. J. Dalton and associates, NCI-406.

Man Years (Calendar year 1960):
Total: 1 2/3
Professional: 1/3
Other: 1 1/3

Project Description:

Objective: As one part of a large collaborative study to determine the role of viruses as etiological agents for some spontaneous tumors, this project is dedicated to the utilization of cultured cells as a substrate for the isolation and subsequent characterization of viruses.

Methods: 1. Tissue culture studies were begun 2 years ago in cooperation with Dr. Robert B. Couch, Surgery, NCI, as reported in project NCI-416(b), 1959. Due to the lack of animal facilities, tests for the presence of virus

were restricted to visible cytopathic changes in vitro. The technique involved the seeding of different cell lines with viable tumor tissue obtained from surgery. In some instances, tumor tissue was obtained at autopsy. Whenever possible, serum was obtained from tumor donors and stored pending serological studies. Fluids and cells from original cultures as well as from blind passage cultures were stored in a freezer for future evaluation.

2. The present investigation was restricted to extracts of human tumors available through the group program, project NCI-420(f). Pellets sedimented from tumor extracts by high speed centrifugation were resuspended in 2.0 ml balanced saline. A portion of the suspension was frozen for reference. The remainder was inoculated into test tube cultures of a variety of cell lines. Each tube received tumor extract equivalent to about 0.03 gm tumor tissue. The cell lines used included human skin, human bone, and monkey kidney cultures obtained from Dr. Wilton Earle; a human synovial cell line obtained from Dr. C. M. Pomerat of the University of Texas; and human embryo fibroblasts routinely supplied by Microbiological Associates. Human intestine cultures from the latter source previously used in this program were discontinued.

Control and inoculated cultures were incubated for at least one month at 37° C. At this time culture material was injected into newborn BALB/c mice, and fresh tissue-cultures were inoculated. These passage cultures were incubated for 2 weeks at 37° C. A portion of each passage culture was injected into newborn mice. The remainder was frozen for future reference.

Nucleic acid extracts prepared from tumors by Dr. Moloney were evaluated in similar fashion. Further studies to be made on selected stored materials include additional passage in vitro combined with staining and serological studies.

Significance: The present investigations provide a foundation for further study and development of techniques to aid in the determination of the relationships of viruses to human cancer.

Proposed Course: The studies outlined will be continued and extended as desired modifications based on exploratory investigations with other tumor viruses are developed.

Part B Included: Yes No X

Serial No. NCI-416(c)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The application of tissue culture to the study of tumor viruses. III. Maintenance of a leukemia virus of mice in vitro.

Principal Investigator: Robert A. Manaker

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1960):
Total: 2 1/3
Professional: 1/3
Others: 2

Project Description:

Objectives: To extend previous investigations on the in vitro propagation of the mouse leukemia virus isolated by Dr. J. B. Moloney, NCI.

Methods: The experimental pattern comprised: (1) Development of a culture system permitting the propagation of mouse spleen cells for extended periods. (2) Determination of the conditions permitting the propagation and maintenance of the Moloney leukemia virus in serial in vitro passage. (3) Establishment of a laboratory mouse spleen cell line to permit in vitro maintenance of the leukemia virus without periodic recourse to animal sources of susceptible tissue.

In the absence of definitive cytopathogenic activity

by the leukemia agent under the conditions employed, all tests for the presence of virus were made in mice. Results were available 4 to 8 months later.

Major Findings: 1. Thirty per cent human serum supplementing a basal medium containing Difco yeastolate, glucose, and Hank's salts from which sodium bicarbonate was omitted proved most useful for the routine initiation of mouse spleen or thymus cultures on glass. When cells were established on the glass, the fluid was usually replaced completely 3 times each week. The reaction of culture fluids overlying older cultures was controlled by the judicious addition of sodium bicarbonate.

2. Primary normal mouse spleen cultures were infected by addition of cell-free extracts of spleen, thymus, and lymph node pools obtained from leukemia mice. After incubation at 37° C for 2 or more weeks, the fluids overlying the cultures were infective for both animals and fresh mouse spleen cultures. Fluids from cultures incubated for shorter periods were not suitable for reliable transmission of virus.

3. Mouse spleen cultures infected with the mouse leukemia agent were maintained without transfer for 3 months. The presence of virus at the end of this period was ascertained by animal response to injected fluids. In these carrier cultures, virus is, therefore, continuously produced without apparent injury to the parasitized host cell.

4. Tissue culture fluids containing leukemia virus may be stored at -70° C and used to infect fresh cultures at a later date.

5. Leukemia has not been observed in mice inoculated with control spleen culture fluids. On the other hand, leukemia has been observed in uninoculated mothers and in uninoculated litter mates of mice infected with active virus material. This suggests transmission of the agent per os.

6. Mouse spleen cultures composed of a mixture of ameboid and connective tissue elements have been maintained for more than a year. During this time, such long-term cultures were infected with the Moloney virus. The virus did not proliferate as rapidly as it did in primary spleen cultures. This was evidenced by the lack of infectivity of samples which were animal tested after 3 weeks of incubation. When incubation was continued for 3 months the fluids from the infected cultures did produce leukemia in test animals. Control culture fluids were without effect. These

results suggest a relatively slow increase in the number of cells producing virus. Eventually the rate at which virus was produced was sufficiently high to surpass the minimum levels of sensitivity of the animal test system.

Significance: The in vitro behavior of the Moloney leukemia virus has been found to be similar in most respects to certain other animal viruses.

Proposed Course: Exploratory investigations will be conducted to develop methods applicable to the demonstration of Moloney leukemia virus activity in vitro.

Part B Included: Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Manaker, R. A., Strother, P. C., Miller, A. A., and Piczak, C. V.: Behavior in vitro of a mouse lymphoid leukemia virus. Accepted for publication in J. Nat. Cancer Inst. 25: 1960.

Honors and Awards relating to this project: None

Serial No. NCI-416(d)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The application of tissue culture to the study of tumor viruses. IV. Indigenous viral contaminants complicating tumor-virus studies in mice.

Principal Investigator: Robert A. Manaker

Other Investigators: None

Cooperating Units: None

Man Years (Calendar year 1960):
Total: 2
Professional: 1/3
Other: 1 2/3

Project Description:

Objectives: The primary objective is to obtain sufficient information concerning the behavior of indigenous mouse viruses to permit recognition of their interference in the course of tumor virus studies. A second objective is to investigate viruses producing latent infections as potential models of tumor virus behavior. A third objective is the control of such agents in the experimental animal colony.

General Statement: Two viruses producing hepatitis and an agent responsible for chronic pneumonia in BALB/c mice were isolated. The characteristics determined for these agents provides the substance of this report.

Methods: 1. Acute hepatic necrosis was frequently observed in weanling BALB/c mice following whole-cell inoculation of the Moloney leukemia. Livers bearing lesions harvested from one group of animals were extracted with saline. The disease was transmitted in weanling mice by filtered extracts. The responsible agent could be propagated in tissue-cultures of Earle's cloned mouse liver (ML) cell line. For purposes of identification, this agent was called MHV-A59.

2. A group of suckling mice with acute hepatitis 3 weeks after inoculation of cell-free Moloney leukemia virus was the source of another agent, termed MHV-B59. The disease could be transmitted to suckling mice by means of filtered extracts of affected livers. Serial passage in weanling mice was not successful. The agent could not be established in the ML cell line.

3. Extracts of the lungs of adult BALB/c mice with chronic pneumonia were introduced intranasally into weanling animals. Three months later, the disease developed in the inoculated mice. Beginning with the extract of the lungs of these animals, the responsible agent was established in serial animal passage. The intranasal instillation of filtrates obtained by passage of lung extract through Selas candles of 03 porosity or Millipore filter discs of type HA produced the disease syndrome within 4 to 7 days. This agent was called CPM-C59.

Major Findings: 1. The behavior of the MHV-A59 agent suggested a virus different from previously described murine hepatitis viruses. MHV-A59 produced acute liver necrosis and some splenic necrosis following intraperitoneal injection of suckling or weanling mice. No cellular inclusions were noted. The hepatic disease was also serially transmissible by the intracerebral route. Suckling and weanling mice of strains BALB/c, Swiss, and C57Bl were most susceptible. Some resistance was exhibited by strain A/LN mice. Strain C3H mice were most resistant; about 50 per cent of both sucklings and weanlings inoculated responded with hepatic disease. Suckling hamsters and suckling Fischer rats showed no evidence of disease following inoculation by the intraperitoneal and intracerebral routes. Ten blind passages of the virus on the chorioallantoic membrane or in the yolk sac of the embryonated hens egg did not yield signs of virus activity.

The virus passed through bacteria-tight filters. No growth was produced on PPLO agar plates. Penicillin, streptomycin, and aureomycin were without effect. The virus survived 18 hours' exposure to anesthetic ether at 4° C. The temperature of 56° C inactivated the agent within 30 minutes. The virus was sedimented by high-speed centrifugation with little loss of activity. Concentrated virus preparations prepared from sedimented pellets did not agglutinate human type O red cells.

The A59 hepatis virus was carried in vitro through 30 serial passages in a cloned mouse liver cell line obtained from Dr. Wilton Earle and through 10 serial passages in primary mouse spleen cultures. Virus activity in both types of cells resulted in the production of syncytia. Carrier cultures have been produced in the mouse liver cell line. The destruction of stock mouse liver cells by virus contained in the nutrient fluids overlying carrier cells that had been cultivated for a year following infection indicated the enduring nature of the host-virus relationship that had been established.

The in vitro techniques developed permitted quantitation of the A59 virus. In addition, neutralizing activity of serum could be determined. By this means, a close relationship to the hepatitis virus isolated by Nelson (Nelson's PRI virus) could be demonstrated. Other hepatitis viruses that were investigated did not grow in the mouse liver cell line. The MHV-1 agent of Gledhill and Andrewes was chosen to represent the latter group. In vivo cross-neutralization tests showed that serum from mice that recovered from infection with the A59 agent neutralized the homologous virus, but not MHV-1. Serum from mice that recovered from infection with MHV-1 had little activity against the homologous virus, but did show some activity against the A59 virus.

2. A second isolate, MHV-B59, responsible for hepatitis in our experimental BALB/c mice, differed from the A59 agent. It was not stable to ether at 4° C, nor was it active in cultures of the mouse liver cell line. A low virulence for weanling mice further served to differentiate it from the A59 virus. Since it resembled Gledhill's MHV-1 in these respects, no further investigations were made. Latent infections in the same BALB/c mouse stock by two hepatitis viruses illustrates the magnitude of the problem of unsuspected virus contamination.

3.. Preliminary investigations were made on a filterable agent responsible for chronic pneumonia in BALB/c mice. Intranasal instillation of low dilutions of lung extract produced the disease in BALB/c mice within a week. Infectivity was destroyed at 56°C within 30 minutes. In strain BALB/c mice, the induction period was related to the dilution of the inoculum and to the route of inoculation. Although a concentrated inoculum could produce the disease in strain A/LN mice, these animals were highly resistant. Aureomycin failed to control the experimental disease in BALB/c mice. Fluids from a variety of cultured cells infected with lung extract were infective for BALB/c mice, indicating that the agent could be maintained in vitro. Its behavior suggested PPLO. Infectivity was lost during an attempt to carry the agent in PPLO broth culture medium, and no conclusions were reached concerning its nature. Frozen samples of infective extracts have been stored for future studies.

Significance: These studies extend the available information on murine viral hepatitis. Isolation of the agent responsible for chronic pneumonia in BALB/c mice permits controlled studies on this disease.

Proposed Course: As time permits, this program will be continued for the purpose of accumulating required information on the presence, behavior, and control of interfering viruses encountered in the course of tumor virus studies.

Part B Included: Yes No X

Serial No. NCI-420 (a)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies on Rous sarcoma virus. Investigations on host response to the virus.

Principal Investigator: W. Ray Bryan

Other Investigators: Dorothy Calnan, John P. Kvedar

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1 1/3
Professional 1/3
Other 1

Project Description:

Objectives: To determine the ultimate limit to which the concentration of virus in Rous sarcoma tissue can be increased through continued selective serial passage in chickens.

Methods: This study has been in progress for several years and details of methodology have been given in previous reports under this same designation. At each virus passage 40 chickens are inoculated and the tumor which appears first, and develops most rapidly, is used as a source of virus for the next passage. The tumors are harvested on the 5th or 6th day after appearing and are stored in a CO_2 box until used. During the past year only one virus passage was made each month, instead of at biweekly intervals as in previous years.

Major Findings: This study has been in progress for approximately 5 years. During this period the virus yield of tumors has been increased about 100 fold by the selective passage procedure described. This is the same as reported last year and it seems apparent that a limit has now been reached, beyond which no further increase in average yield of virus may be expected. Although the 12 points added during the past year indicate a leveling off of virus content, this conclusion is not yet statistically valid and more points will have to be added.

Significance: The chief objective of this project has been a practical one, namely to increase the virus yield of tumor tissue to the point where purification of the virus in appreciable amounts will be feasible. As pointed out last year, the level already reached makes purification feasible, but it was hoped to facilitate purification by further increasing the yield. Lacking evidence that further increases can be expected, studies have already been initiated, in collaboration with Dr. Sober of the Laboratory of Biochemistry, to purify this virus in milligram lots.

Further course of project: This specific project will be terminated as soon as the data show conclusively, by statistical analysis, that the upper limit of virus concentration of tumor tissue has been reached. (About 6 to 8 months will be required with one passage per month.)

Subsequent studies will be pursued under a new project title "Purification of the Rous Sarcoma Virus," in collaboration with Doctors Sober and Rauscher.

Part B included: Yes X No

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Bryan, W. R.: Problems in viral-tumor therapy. Nat. Cancer Inst. Monograph No. 4: 363-376, 1960

Honors and Awards relating to this project: None

Serial No. NCI-420 (f)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Search for viral etiological agents among human and other mammalian tumors not now known to be virus induced.

Principal Investigator: W. Ray Bryan
(Director of broad group program)

Other Investigators: M. A. Fink, R. A. Manaker, J. B. Moloney, F. J. Rauscher, Dorothy Calnan, John P. Kvedar.

Cooperating Units: H. B. Andervont and associates - NCI-400.
A. J. Dalton and associates - NCI-406.

Man Years (calendar year 1960):
Total: 8 3/4
Professional: 3
Other: 5 3/4

Project Description:

Objectives: The primary objective is that of determining whether viral etiological agents are responsible for some spontaneous tumors of humans. Toward this end parallel investigations are conducted on spontaneous tumors of laboratory animals that have not yet been shown to have a viral etiology.

General Statement: This project is part of a large collaborative study. It involves five independent investigators and two research assistants of professional grade as well as supporting personnel within the Virus Oncology Section.

The activities of this Section provide the nucleus of broad collaborative studies involving other sections of the Laboratory of Biology, other laboratories of the National Cancer Institute, and two Public Health Hospitals. Coordination between sections and laboratories is provided by the Chief of the Laboratory of Biology. Over-all direction and coordination of activities involving PHS hospitals is provided by the Assistant Director, National Cancer Institute.

Methods Employed: Since tumor viruses of laboratory animals are

under ordinary conditions species specific, human tumor viruses, if they exist, may also be expected to be species specific. This constitutes the major obstacle to an all-out attack on the human problem with existing knowledge and techniques, since biological tests for viruses cannot be carried out within the same species.

Approaches Currently Being Pursued

(1) Tissue culture studies: Five different types of human cells maintained in tissue culture are being used by Dr. Manaker (see NCI-416) to test for the presence of viral agents in human cancer tissues. Both cell-containing and cell-free preparations of human cancer tissues are inoculated onto tissue cultures of the different types. Observations are made for the detection of cytopathogenic or other changes which might indicate a viral effect on the indicator cultures. Fluids from such cultures as well as from parallel control cultures are inoculated into newborn mice as a further potential test, whether or not tissue-culture changes are observed. Also, blind passages by means of culture fluids are carried out and similar observations and animal inoculations are made through 2 or more blind passages.

Correlated studies involve a search for other methods for demonstrating the presence of viral agents in the absence of overt cytopathic changes, e. g., by specific staining reactions. Systematic changes in culture conditions of known viruses, such as temperature of incubation and type of culture medium, are also being studied in an effort to find optimal conditions for the detection of viruses.

Should viral agents be found in tumor tissues, the problem remains of identifying them as "passengers" of a known type or of establishing their etiological relationship (see under "Proposed course").

(2) Chorioallontoic membrane and allantoic cavity studies: Tests comparable to those in tissue culture (1) are also being conducted on chorio-allantoic membranes and in the allantoic sacs of developing chicken eggs by Dr. Rauscher (see NCI-411). Exploratory tests are being conducted on Japanese quail eggs, and, when a source of supply of adequate numbers of quail eggs becomes available, routine tests will be carried out in these eggs also. Observations are made for localized lesions, or pocks on the originally inoculated membranes, and during several blind passages, starting with extracts of the original membranes. The inoculation of extracts of such membranes into newborn mice is not now being done routinely, but routine tests will be included when sufficient animal room space becomes available.

In the search for additional criteria of the presence of viruses in human tumor tissue, the phenomenon of viral interference is being investigated in embryonated eggs by Dr. Rauscher. Groups of eggs are inoculated first with candidate extracts of human cancer, and one hour later challenged with an inoculum of a known chicken or human virus which grows readily in the embryonated egg. Viral growth in control eggs, but not in eggs previously inoculated with human material would indicate a possible interfering virus. Follow-up studies would be necessary, however, to establish this fact. (See Rauscher's report NCI-411).

(3) Primary inoculations into newborn mice (Bryan, Calnan, Kvedar): Microsome fractions derived from extracts of human cancer tissues are inoculated into newborn mice in tests comparable to those that have been reported by Grace and collaborators, Schwartz and collaborators, Bergolts, and others. Except for a single experiment in which the exact procedures described by Schwartz were used (other than the strain of mouse), the methods of processing and of deriving microsome fractions have been the same as those found most suitable for the Rous sarcoma virus. (See previous reports from this laboratory.) The microsome material is inoculated subcutaneously into BALB/c mice less than 18 hours old. All mice in this study are kept in a separate room used only for such human cancer studies. Routine tests for detection of polyoma virus are carried out on all mothers of litters which are brought into this room (by Mr. Melroy, under the direction of Dr. Fink). Observations are made twice daily (once on week-end days) for ill or dead animals. Beginning 3 months after inoculation, each animal is examined at least once each week for the presence of tumors. All dead animals and animals sacrificed because of illness are carefully autopsied and sections are taken of definite or suspicious lesions.

(4) Tests on nucleic acid fractions derived from human cancerous tissues: Dr. Moloney (see NCI-410_ is studying nucleic acid fractions, derived by phenol extraction, from pellets of microsome material separated by differential ultracentrifugation. The nucleic acid fractions are inoculated intracerebrally into some, and subcutaneously into other newborn mice. The mice are housed in the same isolation room as mentioned under (3), for human studies. Routine examinations and autopsies are carried out in similar manner.

Similar studies on the nucleic acid extracted from cell nuclei and from whole cancerous tissues will be included when sufficient tumor material and adequate animal room space become available.

(5) Use of procedures for producing immunological tolerance in tests for human viruses involving the inoculation of other animal species: Preparatory to the testing of human materials in laboratory animals rendered immunologically tolerant to human antigens, studies are being conducted by Dr. Fink (see NCI-415) with a known tumor virus (the Rous sarcoma virus) and a foreign recipient species (the mouse). If satisfactory techniques can be developed with which this virus can be made to cross species barriers, they will be employed for the routine testing of candidate human cancer extracts in mice and other laboratory animals.

(6) Electronmicroscopic studies (in collaboration with Dr. Dalton and associates (see NCI-406)): For each human cancer extract tested, two pellets of the microsome fraction sedimented by ultracentrifugation are turned over to Dr. Dalton for embedding and study by thin-section electronmicroscopy. Although embedded specimens of all cancers thus far investigated are on hand, the actual cutting of sections and examination under the electronmicroscope have not yet been initiated on a routine basis, due to a lack of sufficient personnel and facilities.

Major Findings

The tissue culture studies have now been in progress for about two years. No evidence has been obtained thus far of a virus of any type being associated with human cancer tissue. A total of 75 separate cancers have been investigated in tissue culture.

The studies involving other approaches were initiated only last May, and the first group of test animals has therefore been under observation for only 5 months. The majority of them have been under observation for only 2 to 4 months. This is an insufficient time for the development of tumors. The absence of positive results at this time is therefore without significance. A total of 10 cancer specimens have been employed in coordinated experiments involving all or most of the various approaches listed. An additional experiment with human leukemic brain tissue obtained at autopsy was carried out under the exact technical conditions described by Schwartz.

Significance

The need for an all-out effort to determine whether viruses might be etiologically related to human cancers is now generally recognized. The present investigations are directed toward this end. The approaches described are those most accessible for immediate pursuance with available facilities and personnel.

Proposed Course of Project

The experiments outlined will be continued with present techniques until adequate time has elapsed, and until adequate data have been acquired to determine whether the results are decisively positive or negative. At this time modifications in techniques will be introduced or a particular approach may be dropped altogether. In the meantime exploratory investigations will be carried out by each of the investigators mentioned on possible modifications of procedures, or on new avenues of approach. Each of the participating investigators is now working with one or more laboratory model systems involving a known tumor virus. In addition to systematic studies on these systems, which may be expected to be definitely productive, considerable effort is being directed toward the development of methods and new approaches which may be applicable to the human studies.

Many ancillary problems accompany the main problem of looking for human cancer viruses. One of the most important of these is the identification of "passenger" viruses which may turn up in human cancerous tissues. Toward this end a viral diagnostic laboratory is being planned which will enable us to identify not only all, or most of the known human viruses, but also viruses which may be latent in various laboratory animal strains and species. The latter will require considerable research on animal viral flora, and the development of suitable diagnostic sera and antigens.

Another ancillary problem involves the development of test animal colonies that are either free from specific viruses which infect the species, or are identified with respect to latent viruses which they carry. Research toward these ends is already in progress.

When facilities become available at the new NIH farm, it is planned to establish a large breeding and laying flock of Japanese Quail. Not only are the eggs needed in large numbers for routine testing of cancer extracts, as already described, but a new approach will be introduced which may permit the detection of certain types of human cancer viruses. The human tissue extracts and microsome fractions will be introduced into developing quail in the embryonic stage, and the quail will be allowed to hatch. The latter will then be kept for 2 or more years to determine whether tumors will appear. The small size of the adult quail makes feasible the long-term holding of large numbers of these test animals for such studies, which would be impractical with chickens.

This technique might permit detection of agents having very long latent periods, provided that the viral antigens can be made to cross species by injecting the hosts during their embryonic life. (See Rauscher's report NCI-411.)

Animal tumors not now known to be associated with viral etiological agents will be "put through the same paces" as those employed or proposed for investigating human tumors. This will provide a means of biological testing of any agent revealed within the same animal species, thus verifying the adequacy of the techniques.

Part B Included Yes X No

Serial No. NCI-420(f)

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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Bryan, W. R.: A reconsideration of the nature of the neoplastic reaction in the light of recent advances in cancer research. J. Nat. Cancer Inst. 24: 221-251, 1960.

Honors and Awards relating to this project: None

Serial No. NCI-420(g)
1. Laboratory of Biology
2. Virus Oncology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Purification of the Rous sarcoma virus.

Principal Investigator: W. Ray Bryan, H. A. Sober

Other Investigators: F. J. Rauscher, J. P. Kvedar.

Cooperating Units: Laboratory of Biochemistry, NCI.

Man Years (calendar year 1960):
Total: 2
Professional: 2/3
Other: 1/3

Project Description:

Objective: To achieve high purification of the Rous sarcoma virus in sufficient quantities so that basic studies may be made on the nature, properties, and activity of the virus and of its constituent nucleic acid.

General Comments: This is a new project initiated during the past year. As indicated in the accompanying report, NCI-420(d), the virus content of Rous sarcoma tissue has been increased during recent years to the point where achievement of the above stated objective seems reasonable and practicable.

Methods: Purification is sought through differential centrifugation in an ultracentrifuge, and ion-exchange chromatography employing cellulose columns. Preparations are analyzed for viral activity by the chorioallantoic membrane-pock counting technique. Chemical analyses include determinations of nitrogen, phosphorus, optical

density, etc. Conditions necessary for the stability of the virus are being studied (e.g., buffer salts, aggregation proclivities, etc.).

Major Findings: As described in another report (NCI-420(d)) extracts of tumors in duced by a "high potency" passage strain of Rous sarcoma virus contain about 100 times more virus activity than extracts of tumors induced with the strongest doses of standard Rous sarcoma virus. Studies on purification of the virus from such "high potency" tumors show that:

(1) A similar 100 fold increase in virus activity is present in partially purified fractions separated by differential centrifugation and cellulose chromatography.

(2) By both of these procedures there is a 2 to 3 fold increase in viral activity per gram equivalent of tumor tissue, indicating that an "inhibiting" factor of some type is removed.

Significance to bio-medical research: The nature of tumor viruses and the manner in which they bring about the transformation of normal cells to tumor cells constitutes one of the most fundamental problems in cancer research. Knowledge of their physico-chemical properties as well as the availability of practical working quantities of purified virus are essential for basic studies on their mode of action and chemical composition. The goal of reaching adequate quantities of the purified agent now appears to be achievable with the Rous sarcoma virus, which has been under laboratory investigation toward this end for many years. Methods applicable to this virus may be applicable also to other tumor viruses, including those of humans. Knowledge of the mode of action of this virus (e.g. through enzyme induction by viral nucleic acids, or through immuno-chemical reactions of viral proteins) will undoubtedly throw light on the mode of action of other tumor viruses as well.

Proposed Course of Project: Since the project was initiated only recently, systematic exploratory studies of the type described will be continued during the coming year. Also investigations will be carried out to determine the nature of the "inhibitory" factor that has been disclosed.

Part B Included Yes No X

Serial No. NCI-250
1. Lab. Chemical Pharmacology
2. Office of the Chief
3. Bethesda 14, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A

Project Title: Planning, Management, and Coordination of Investigations, Organized on a Multidisciplinary Team Approach, on Basic Science Aspects of Pharmacology for Cancer.

Principal Investigator: Murray J. Shear

Other Investigators: Staff of Laboratory of Chemical Pharmacology.

Cooperating Units: These are listed in the accompanying Project Reports.

Man Years (calendar year 1960)		Patient Days
Total:	12	None
Professional:	3	
Other:	9	

Project Description:

A. General

I. Plan

The over-all philosophy envisages a dual approach in contributing basic knowledge aimed at the chemical control of cancer. Major phenomena in malignant disease, such as:

(1) conversion of normal cells to malignant

(2) growth of these microscopic foci to detectible tumors

(3) invasion of adjacent normal tissues

(4) spread to other sites

(5) the rare "spontaneous" regression

are all viewed as conceivably susceptible to the influence of chemical agents. The usual concept of pharmacology, as the science which deals with the effects of extrinsic drugs, is here broadened to include endogenous materials, especially the macromolecular, which constitute Host Defense Factors.

Chemotherapy, which was pioneered by my group when the climate was unfavorable, is now not only respectable but even popular. However, the vast expansion of empirical screening and of empirical therapeutic trials in patients, have so far yielded only modest progress, e.g., the newer drugs have all been limited in their scope and effects in the clinic, under the restraints imposed by their toxicity at useful dose levels, and by the appearance of resistance in tumors that were sensitive at the outset.

The need is therefore clear for a greater effort in basic science for chemotherapy to accompany the large enterprise in empirical work in chemotherapy itself. This calls for a greater understanding of various mechanisms of action as regards: anti-cancer effects; the many undesirable effects on normal tissues and functions which are commonly lumped together as "toxicity"; and other Host-Tumor-Drug interrelationships.

As a temporary scaffolding for use in designing other experimental approaches, one of our working hypotheses is what I have termed the "buffer systems" of the mammalian host. According to this notion: the mammalian body contains substances and processes some of which promote, and some of which retard, the phenomena in malignancy listed above; when the balance between these postulated opposing influences is disturbed sufficiently, effects on malignant phenomena become evident; and, with adequate basic knowledge, attempts to manipulate these endogenous factors would become feasible.

Of course, faith in the existence of such postulated factors is not widespread. The situation might be compared by some to that of the Blind Man in a totally Dark Room, Looking for a Black Cat--that isn't there! But since fresh approaches are sorely needed, we are prepared to gamble our time and efforts. To quote from a Book That I Have Not Yet Written:

> "The difference between a Brilliant Idea and a Silly Idea:
> A Silly Idea is a brilliant one that was not supported when the research was done.
> A Brilliant Idea is a silly one that was established by the research that it suggested."

If the search for Host Defense Factors is rewarded by the finding of endogenous materials which are useful in controlling the continued growth, and spread, of cancer tissue, a valuable adjunct would be provided to surgery, to radiation, and to anti-cancer drugs whose effects at non-toxic levels might thereby be enhanced.

Research with conventional types of drugs obviously comes under the purview of a department which bears the label of "Chemical Pharmacology". But what of the body's own macromolecules--of antigens, of antibodies, etc.? In the first place, immunologically-active substances are no longer regarded as being endowed with mystic qualities. Immunological reactions are but special instances of interactions between particular chemical substances with macromolecular properties. Does the study of such materials and of their effects on the host, with the aim of influencing malignant processes, constitute a misuse of the term "Chemical Pharmacology"? Not if one realizes the implications of the satiric definition of a drug as "Any chemical agent which, administered to any living system, results in a publishable paper"!

Space will not be taken here to marshall the various types of evidence which have led us to suspect that this kind of approach--the search for Host Defense Factors--may be a fruitful one. The kinds of basic knowledge that emerge may well be useful for several of the major objectives in cancer research. For example, the many attempts to develop, by frontal attack, a reliable diagnostic procedure for early cancer have all led to disappointment. Desirable as is the attainment of this objective, the likelihood of success by further frontal attack is remote. On the other hand, long-range study of Host Reactions to the Presence of Small Amounts of Malignant Tissue would probably uncover other host responses in addition to those few which are already known. As these emerge, they would be available for examination as to possible value in early diagnosis.

Among the many situations in which factors in the host play an important role, I might mention two at this point. One is the disturbance produced by surgery, as illustrated by the stimulation of dormant tumor cells by surgery performed in non-tumor-containing tissue. Another is the nature of the host factors which promote, or oppose, the transformation by carcinogens of normal cells to malignant. As we pointed out many years ago, "carcinogenic potency" is not an intrinsic property of the chemical agent, but merely an abbreviated summary of the result of the interaction of the chemical and a particular biological system. Since certain tissues of certain mammalian species respond readily with tumor genesis to a given chemical while others do not, the difference must reside in the host itself. Greater understanding of these postulated factors would make possible rational approaches to Prevention.

II. Organization

This Laboratory continues to have five organizational units: four Sections and the Office of the Chief. The turnover of personnel continues to be greater than we consider desirable. During the year, 14 persons were recruited to fill vacancies, as Guest Workers and Fellows, and as replacements. As opportunity arises (or is created) to replace personnel, we make every effort to have the change contribute to the strengthening of the research program and to raising the caliber of the staff. I need not repeat here the well-known difficulties which confront us in recruiting and retaining first-class workers in an era of shortage of scientific personnel and the consequent competition for the best.

The Office of the Chief continues to provide the Laboratory with centralized services such as: procurement and care of laboratory animals; maintenance of stocks of various transplantable tumors; continuous furnishing of mice bearing transplanted tumors of the required type and age, and in the necessary numbers; maintenance of property and property records; common laboratory service rooms; clerical help; etc.

We have recommended that the name of the Section headed by Dr. Landy be changed from "Polysaccharide" to "Immunology". Although polysaccharides continue to be useful tools in the studies of this Section, the vistas opened up by their employment call for a name which better designates the broadened scope of that program. The accomplishments of Dr. Landy and his group, set forth in his accompanying Project Report, have been outstanding. My evaluation is provided in the recent documents recommending his promotion to Grade 208g.

Dr. Goldin continues to manage with great effectiveness two major enterprises--basic researches in the Biochemical Pharmacology Section, and the Drug Development Program for which he is the Scientific Project Officer (a CCNSC contract operation at Microbiological Associates, Inc.). The latter activity is closely co-ordinated with the needs of the NCI Clinical Chemotherapy Program by intimate working relationship with the NCI Clinical Director and his staff. Details of accomplishments are given

in the accompanying Project Report of Dr. Goldin. My own comments and evaluations are set forth in the recent documents recommending promotion of Dr. Goldin to Grade 208g.

Dr. Mora displays imagination, enterprise, and vigor in the planning and execution of the program of the Macromolecular Chemistry Section, and in recruitment of capable young people. Here, too, details are provided in his accompanying Project Report. My evaluation is given in the recent documents recommending his promotion to Grade GS-15.

Increasing use is being made of living cells (e.g., ascites tumors, tissue cultures of normal and malignant cell lines of animal and of human origin) in the various projects of the three Sections mentioned above. These are provided by Dr. Belkin and his assistants in the Cellular Pharmacology Section. Dr. Belkin collaborates with the other scientists to the limit of his available time; for the rest, he generously provides materiel, information, and advice from his great store of knowledge and experience with living cells and the effects of chemical agents upon them. Men from the other Sections are thus able to make fuller utilization of the space and facilities nominally under the jurisdiction of Dr. Belkin's Section. Still greater utilization is projected. For example: in the immunologic characterization of mammalian cells being conducted by Dr. Landy's group, both tissue culture material and ascites cells are employed; now that Dr. Mora's group has made good progress in his studies with the "model" system of bacteriophage and bacterial cells, the concepts and techniques are being extended to a tumor-producing virus and cultures of mammalian cells (cf. plaque technique).

Headway is being made in still other ways in the cross-fertilization of ideas, interchange of materiel and facilities, and of dove-tailing of research across Section lines. The fine work on endotoxin-detoxifying components present in blood serum and liver, accomplished in Dr. Landy's Section, warrants the attempt at isolation of these enzymes. Dr. Mora and his group are getting set up for this intimately-related line of work, and are exploring various <u>in vitro</u> techniques for a rapid and convenient assay procedure.

Another example is the projected incorporation of anti-cancer drugs in synthetic macromolecules. The work is jointly planned by Dr. Mora and Dr. Goldin. The synthetic work is to be done in the Section of the former, and the animal experiments in that of the latter; a beginning has been made in this direction.

Still other examples are Dr. Goldin's developing interest in immunology and in carcinogenesis. His own observations have led him to put his head together with Dr. Landy in looking ahead to joint investigations on immunological aspects encountered in drug treatment of tumors. He also plans to develop, in collaboration with the Office of the Chief, a carcinogen-induced tumor system for screening chemicals against primary tumors; in this he is encouraged by Prof. E. K. Marshall.

The foregoing illustrations exemplify the intra-organizational relationships in the working programs of the different Sections in this Laboratory. In this regard these cross-section interrelationships appear to be closer than in other NCI laboratories. Each of the programs has retained its individuality, yet overlapping interests have developed so that functional interdigitation has been developing in a natural way. This trend is being encouraged by a variety of devices. These senior scientists work out their missions--separately when advantageous, with other groups when more valuable--in the accomplishment of a common goal.

Bridging the gap between the fundamental findings in the basic science laboratories and the studies in patients in the clinical Branches continues to be a largely unfilled need. In this respect, too, the program of this Laboratory has contributed greatly to the over-all mission of the NCI.

B. Specific

Several items are mentioned here which are not covered in the accompanying four Project Reports of the Section Heads, or in the preceding part of this report.

1. Three of the key men have received offers of interesting posts in consequence of their outstanding work. As yet, however, they have preferred to continue in their present positions.

2. Requests continue to be received by the Chief for consultation, advice, and help in many different kinds of scientific matters. These appeals come from various parts of the NIH, other Federal Government Departments, universities, scientific organizations both academic and industrial, and Embassies of foreign countries. These requests are met within the limits of feasibility.

3. As President of the American Association for Cancer Research, the Chief had to devote considerable time to the management of the affairs of that organization, whose problems had grown increasingly acute because of the growth in the size of its membership.

4. As Chairman of the Chemotherapy Committee of the Internat. Union Against Cancer, the Chief was responsible for the activities of this group on the world-wide scene, organized an International Symposium on this subject in Tokyo (Oct. 7 and 8), and participated in the deliberations of the UICC Research Commission in Osaka, Japan (Oct. 17, 18). While in Japan, gave lectures and participated in seminars and conferences (by invitation) in various universities and institutes in Sapporo, Sendai, Osaka and Tokyo.

5. As Chairman of the Finance Committee of the UICC, the Chief participated in the meeting of its Budget Committee in London in January. He then transacted business, of both Committees which he heads, in Portugal, France, Switzerland, Austria and Hungary. He lectured in Portugal and Hungary, and talked for radio broadcasting in those countries. Special permission was extended by the U.S. Dept. of State to enable him to accept the invitation of Hungarian cancer research workers.

6. At the invitation of a Sub-Committee of the U.S. Senate, and in the capacity of an officer of the Amer.Assn. Cancer Res., the Chief prepared a comprehensive document on the UICC, providing the information on the topics requested. This material is now in press, and galley proofs have been corrected. Acknowledgement was received stating that this material "handsomely fills our needs".

7. Requests continue to be received from leading investigators in various lines of work for supplies of "Shear's Polysaccharide" from S. marcescens. These are provided insofar as feasible.

Future Course:

This has been indicated in a number of passages in the foregoing running commentary. I might add at this point a statement from my report of last year:

> Giving leadership to talented, strong-willed, temperamental scientists is difficult enough when each works on a completely independent

project, but the difficulties are multiplied many-fold when the objective is to maintain their enthusiasm for working toward a common target and with materiel in common. However, it is worth the effort!

Part B Honors, Awards, and Publications

Publications (no abstracts included):

Three articles in which the Chief was one of the authors are listed in the accompanying Reports of Dr. Landy and of Dr. Mora, and hence are not repeated here.

One joint publication had as its authors men from three different organizational units of the Laboratory. This paper, not listed in any of the Section reports, is:

Merler, E., Perrault, A., Trapani, R-J., Landy, M., and Shear, M. J. Absence of Endotoxic Activity in Materials Derived Without Bacterial Contamination from Mammalian Tissue. Proc.Soc.Exp.Biol.Med. (In press).

Honors and Awards pertaining to this project:

1. Elected President of the Am. Assn. Cancer Research for 1960-61.

2. Appointed a member of the 6-man USA National Committee for UICC (an NRC committee).

3. The CBS broadcast on TV on April 21 entitled "History of a Cancer", in which I was interviewed by Dr. Tom Dooley of Laos, evoked a widespread and favorable response.

4. Lectured (by invitation) at various universities here and abroad. Declined, regretfully, the invitation from the Director of the Drug Research Institute, Lucknow, India to give a paper in the symposium on "Advancing Frontiers in Life Sciences" in the Silver Jubilee celebration of the National Institute of Sciences in New Delhi in December 1960.

5. Gave the Main Address at the Dedication of a new 100-bed wing of the Jewish Memorial Hospital, Boston on Dec. 4.

6. Invited to contribute a free-wheeling article, on any subject of my own choosing, to the new journal "Perspectives in Biology and Medicine".

Serial No. NCI-253
1. Laboratory of Chemical Pharmacology
2. Biochemical Pharmacology Section
3. Bethesda 14, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Biochemical and Pharmacological Studies of Host-Tumor Relationships in Tumor Chemotherapy

Principal Investigator: Dr. A. Goldin

Other Investigators: Dr. A. Schrecker, Dr. M. Chirigos, Dr. J.A.R. Mead, Dr. J. Glynn, S. Humphreys, J. Venditti, N. Mantel (Biometry Branch)

Cooperating Units:

(a) General Medicine Branch, NCI: Dr. E. Frei and staff. Serial No. NCI 226.

(b) Laboratory of Biology, NCI: Dr. J. Moloney. Serial No. NCI 420.

(c) Laboratory of Pathology and Histochemistry, NIAMD: Dr. George Glenner, Serial No. NIAMD-LPH-15.

(d) Pathologic Anatomy Branch, NCI: Dr. Louis Thomas. Serial No. NCI 853.

(e) Graduate School, Biochemistry Department, Brandeis University, Waltham, Massachusetts: Dr. Nathan O. Kaplan and staff; Chemistry Department: Dr. Orrie Friedman and staff.

(f) Department of Pharmacology, Tufts University Medical School, Boston, Massachusetts: Dr. M. Friedkin, Dr. R. Kisliuk.

(g) Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri: Dr. R. Burton, Dr. R. Salvador.

(h) University of Miami Medical School, Miami, Florida: Dr. J. Colsky.

(i) Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma: Dr. P. Condit.

(j) Cancer Chemotherapy National Service Center: Microbiological Associates, Inc., Bethesda, Maryland (Contract No. SA-43-ph-2371) Serial No. NCI-216.

Man Years (calendar year 1960):
Total: 12-3/4
Professional: 6-1/4
Other: 6-1/2

Project Description:

Objectives:

1. Study of the mechanism of action of antitumor agents including metabolic antagonists, alkylating agents, antibiotics, etc.

2. Synthesis of new agents.

3. Study of host-tumor interrelationships with respect to drug action.

4. Establishment of new therapeutic principles and procedures in the management of tumor growth in animals.

Methods Employed:

Assay procedures involving methods of inhibition analysis for animals with and without tumors. In vivo and in vitro biochemical procedures. Radioactive tracer techniques. Organic chemistry procedures.

Major Findings:

1. Relationship of Immunity to Chemotherapy (with Chirigos, Humphreys, Venditti): Studies have been continued on the role that host immunity may play in influencing the host-tumor relationship in chemotherapy.

Work has been extended on the influence of folic acid antagonists on the growth of resistant variants of leukemia L1210 in foreign strains of mice, and on the homograft response. Leukemia L1210 and three antifolic-resistant variants (M46R, M66-3R, and C82R) were employed. M46R is resistant to amethopterin (MTX) and relatively cross resistant to 3',5'-dichloroamethopterin (DCM). M66-3R and C82R are resistant to DCM and MTX. We had previously observed that L1210 and its resistant

variants grow, and then regress in typical homograft fashion in foreign strains of mice; this confers immunity to reinoculation. Treatment of the resistant tumors with folic acid antagonists (MTX and DCM) suppressed the homograft response; the resistant leukemias grew progressively and killed the animals.

However, in out-of-strain animals made immune, treatment with folic acid antagonists did not readily overcome the immunity. Also, treatment with the antifolics did not interfere substantially with the development of host immunity. Treatment with a dose level of DCM which suppressed the homograft response, resulting in progressive growth of the C82 antifolic resistant leukemia, failed to interfere with the ability of an inoculum of L1210 or of normal spleen to render the mice immune to reinoculation.

Suppression of the homograft response may not occur if the folic acid antagonist is more potent than the one used to induce resistance. In such a case, inhibition of tumor growth may result. This occurred with DCM treatment in out-of-strain mice of the subline M46R which is resistant to amethopterin.

The above studies show that the observation of abrogation of the homograft response to resistant variants of leukemia L1210 on treatment with folic acid antagonists is not a reflection of a generalized suppression by antifolics of immune responses. It is a different manifestation than the finding that successful therapy of L1210 in-strain results in immunity of the mice. The studies show that such approaches may be useful when properly understood in having immune responses support chemotherapeutic activity of drugs.

2. Combination of Chemotherapy and Surgery (with Chirigos, Humphreys, Colsky): Surgery of Ca-755 mammary adenocarcinoma in-strain (C57 Bl. mice) followed by therapy with 6-mercaptopurine (6-MP), resulted in extensive prolongation of survival time and a high percentage of survivors with no recurrence of tumor. This result was not obtained by treatment with any dose of 6-mercaptopurine or by surgery alone. The favorable effect observed following this combination treatment may be attributable to the reduction of the tumor mass by surgery. Other studies in this Section have shown that, with small tumors (Ca-755), 6-MP is more effective in inhibiting tumor growth, and in increasing the survival time of mice, than with well advanced tumors.

It is possible that host immunity may also contribute to the therapeutic effect of 6-MP. In the tumor-free survivors, reinoculation with Ca-755 failed to result in tumor growth in an appreciable number of cases. Where the reinoculum did "take", tumor growth was frequently retarded.

We had previously shown that successful treatment of systemic L1210 in-strain resulted in host immunity and enhanced therapy of an antifolic resistant subline of leukemia L1210. The possibility exists that, with or without surgery, appropriate drug therapy may so alter tumor cells that they will elicit immune responses in the host. Even if antigenic activity were weak, resultant host immunity could conceivably enhance chemotherapeutic activity. Other tumor systems, including spontaneous tumors, will be investigated employing this methodology.

3. Central Nervous System Leukemia (with Chirigos, L. Thomas (Pathology) and Humphreys): Dr. Thomas' and Dr. Freireich's detailed studies have emphasized that central nervous system involvement is a serious complicating factor in the chemotherapy of clinical leukemia. A similar situation has been observed in mice treated with doses of dichloro- and bromochloro-amethopterin high enough to give extensive increases in survival time with leukemia L1210. Some of the mice developed neurologic symptoms and paralysis of the hind legs. Transplants from the brain usually resulted in tumor growth. In such mice, Dr. Thelma Dunn found CNS and brain involvement.

Model systems were sought for studying the chemotherapy of central nervous system leukemia. A method has now been devised involving intracerebral inoculation of leukemia; it is providing a useful tool for studies on the effect of therapy when there is a high concentration of leukemic cells in the brain, and when the ability of the drug to cross the blood-brain barrier could be of considerable importance. Some of the findings are summarized below.

Intracranially inoculated L1210 in mice showed profuse infiltration in the subarachnoid spaces. Small nodules of leukemic cells were seen in the ventricles, and intracerebral vessels were surrounded by a cuff of leukemic cells. These observations are similar to those observed at autopsy in patients diagnosed as having meningeal leukemia. Thus, where L1210 was inoculated IC, brain tissue was infiltrated and the disease became systemic two to three days following IC inoculation.

Amethopterin and DCM were found to be capable of increasing the survival time of mice inoculated IC with L1210, even when treatment was initiated as late as a few days prior to death of controls. The effectiveness of the drugs suggests that they may be capable of crossing the blood-brain barrier in sufficient quantity to exert direct antileukemic action in the brain.

A detailed study was conducted on the relative effectiveness of Cytoxan in the treatment of subcutaneously inoculated (SC) and intracranially inoculated (IC) leukemia. This drug was chosen because of its marked activity against a wide variety of transplantable tumors and the current clinical interest in it. With therapy initiated early or late in the course of the disease, and with various schedules of drug administration, treatment of the mice with intracranially inoculated leukemia was considerably more difficult than treatment of the mice in which the disease was inoculated subcutaneously. Although Cytoxan did elicit significant extension in life span of mice with IC leukemia, the increases were of limited extent except with early treatment at relatively low inoculum levels of leukemic cells.

A comparison was made of the relative effectiveness of Cytoxan on the transplantability of brain tissue of IC and SC inoculated mice. Brain tissue was removed from SC and IC inoculated mice with advanced leukemia at 1-2 hours after administration of a single dose of Cytoxan, and implanted into other mice. Brain tissue transplants taken from SC inoculated mice failed to produce tumor growth in recipient mice, indicating that Cytoxan was effective in inhibiting the growth of leukemic cells present in brain. However, Cytoxan was not as effective in mice implanted IC with L1210, since 75% of the recipient mice grew tumors when implanted with donor brain tissue. The results provide additional evidence that Cytoxan was capable of crossing the blood brain barrier and also indicate that fewer leukemic cells were present in the brain of SC inoculated mice as contrasted with the IC inoculated animals.

The duration of Cytoxan effect in mice with advanced leukemia L1210 was determined. Eight days after SC implantation of L1210, mice were given a single dose of Cytoxan, and blood and brain were bioassayed. One injection of Cytoxan was effective in inhibiting transplantability for as much as six days.

Another type of experiment showed that therapeutic doses of Cytoxan are present in active form for only 1/2 to 1 hour after in vivo administration. Cytoxan was administered SC at different time intervals into mice implanted IC or SC with L1210. Pretreatment with Cytoxan at one hour prior to L1210 implantation did not produce any increase in survival time. A definite increase in survival time occurred, however, when Cytoxan was administered 1/2 hour prior to L1210 implantation or at a subsequent time.

In another study of schedules of treatment, Cytoxan and DCM were less effective in treating IC inoculated leukemia than intravenously inoculated disease.

Studies are in progress on the effectiveness of other known antileukemic agents in the treatment of mice with IC inoculated and CNS leukemia. As part of a collaborative program, appropriate tissues of treated animals are being provided to Dr. Louis Thomas, of the Pathologic Anatomy Branch, for histologic comparison with treated tissues of patients.

4. Cytoxan Derivatives (with Friedman): The collaborative research program with Dr. Orrie Friedman at Brandeis University, has continued. A number of congeners of Cytoxan, synthesized by him, have now been tested against advanced L1210. Although they were relatively ineffective as compared with Cytoxan in increasing survival time of the mice, they are providing important information on structure-activity relationships and mechanism in this series. This study is being continued in coordination with the interests of CCNSC in this area.

5. Virus-induced Leukemia Studies (with Chirigos, Glynn, Humphreys, J. Moloney (Laboratory of Biology) and Mantel (Biometry): The program has been making good progress. We now have on hand a satisfactory assay system for determining the relative effectiveness of drugs in increasing the survival time of mice implanted with the cells of this virus leukemia. A standardized inoculum kills rapidly, in a relatively narrow time range. Several drugs have now been tested in this system, which will be utilized in the Drug Development and Evaluation contract (SA-43-ph-2371) of this Section. The system is also suitable for studies of the mechanism of action of antitumor agents.

Of the drugs tested to date, Cytoxan, triethylene

melamine, and phenylalanine mustard were the most effective in increasing the survival time of the animals. They showed activity against the transplantable whole cell leukemia on various schedules of treatment, including treatment initiated early or late in the course of the disease. The effectiveness of the drug was shown to be dependent upon the inoculum level and on the stage of the disease. With treatment instituted early, a doubling of the life span has been observed. No survivors of the disease have been obtained to date. Whole body x-irradiation was also effective in prolonging life span.

6-Mercaptopurine, 3',5'-dichloroamethopterin and amethopterin, 5-fluorouracil, and meticortelone showed moderate to no activity in this system.

In preliminary studies with leukemia following inoculation of the virus, minimal increases in survival time were obtained with Cytoxan, triethylene melamine, methotrexate, and methylglyoxal bis-guanyl hydrazone. Reduction in the size of spleen, liver, thymus, and nodes were also observed. However, variability of the controls prevented precise determination of activity. Dr. Moloney has "stepped up" the potency of the virus to the point where we are now in a position to start more definitive testing of drugs with primary virus-induced leukemia. This material will also be suitable for testing the effectiveness of drugs against the virus itself.

6. Other Tumor Systems (with Chirigos and Humphreys):
(a) Hepatoma 129 (from Dr. Belkin). Both Cytoxan and 6-mercaptopurine were effective on a number of regimens of treatment in retarding tumor growth and increasing the survival time of mice inoculated SC with hepatoma 129. The drugs were effective even when treatment was withheld until palpable tumors (10 mm in diameter) were present at the site of inoculation.

(b) Spontaneous mammary tumor in C_3H mice (with Humphreys, Chirigos, Venditti, Mantel). An initial study has been completed testing the effectiveness of 3',5'-dichloroamethopterin (DCM), 6-mercaptopurine (6-MP), and Cytoxan in the treatment of spontaneous mammary tumor in C_3H mice. Appropriate randomization procedures were employed. A daily treatment schedule was followed with dose levels in the optimal dose range for treatment of L1210 (50 mg/kg DCM, 60 mg/kg 6-MP, and 50 mg/kg Cytoxan). The controls showed considerable variability from the day of entry into the experiment to the day of death (median 40 days, range 5-117 days).

It was clear, however, that the three therapies failed to increase survival time. The median survival times and range for the treated groups were: DCM, 36 days (1-158); 6-MP, 26 days (1-60); Cytoxan, 24 days (1-90). The decrease in median survival time with 6-MP and Cytoxan suggests that drug toxicity was limiting at the doses employed.

Although no increases in survival time were observed, each of the three drugs appeared to retard the growth of the tumor, as measured from the day of initiation of therapy.

Additional exploratory studies will be conducted with a view to improvement of methodology in the chemotherapy of spontaneous tumors.

7. Evaluation of Antitumor Agents (with Venditti, Kline, Mantel): Assay procedures for the quantitative evaluation of new compounds of clinical interest have been developed in this laboratory. Three transplantable mouse tumors (L1210, sarcoma 37, and adenocarcinoma 755) have been employed extensively in these studies, conducted primarily in the Drug Development and Evaluation Program (Contract No. SA-43-ph-2371). The wide scope of the Drug Development and Evaluation Program precludes a detailed description in this report of the findings. A more complete resume of the work done in this program is presented in a separate report (NCI-216).

The advanced leukemia L1210 assay system:

213 compounds have now been evaluated for their ability to prolong the life of mice with systemic L1210. The most active compounds against this tumor have been the halogenated derivatives of amethopterin. For example, 3',5'-dichloroamethopterin was about 4 times as active as amethopterin. Extensive structure-activity relationship studies among folic acid derivatives have shown that appreciable antileukemic activity is limited to the 4-amino derivatives of this metabolite. However, substitutions in various other positions (e.g. alkyl substitutions in the 10 position or halogenation in the benzene ring) can profoundly alter the degree of antileukemic effectiveness among the 4-amino derivatives. Cytoxan, a cyclophosphoramide nitrogen mustard, displayed activity equal to that of amethopterin. Uracil mustard and a number of ethyleneimines showed moderate antileukemic activity, but were only about 50% as effective as Cytoxan. Of thirty-five purines tested against advanced L1210, none appeared to be more effective than 6-mercaptopurine, which

was 50% as active as amethopterin. Among the pyrimidines, 5-fluorouracil and its riboside displayed about equal effectiveness (about 40% of the amethopterin effect). 4-Aminopyrazolo(3,4-d)pyrimidine was about 25% as effective as amethopterin. None of the various derivatives of this compound showed greater activity. Good activity was seen among the methylglyoxalbisguanyhydrazones. Three such compounds tested displayed activities of about 65%. Glyoxalbisguanylhydrazone, itself, was relatively inactive. Among the antibiotics, E-73 acetate, Streptovitacin A, and Actinomycin D were the most active, being about 30% as effective as amethopterin.

Special studies with the advanced L1210 assay system have been directed toward investigations of factors which may alter the host-tumor-drug relationship, including the influence of the route of administration, schedule of treatment, effect of therapy with drug combinations, etc. It has been shown, for example, that the efficacy of therapy with 3',5'-dichloroamethopterin or 3'-bromo-5'-chloroamethopterin is substantially reduced when the drug is given orally. Consequently, the superiority of the halogenated derivatives over amethopterin, seen on subcutaneous administration, disappears when the drugs are given via the oral route. On the other hand, oral administration did not seriously reduce the effectiveness of 6-mercaptopurine. The methylglyoxalbisguanylhydrazones were only slightly less effective orally than subcutaneously. Studies of the influence of the treatment schedule on antileukemic activity showed that Cytoxan was more effective when given weekly than when given daily, twice daily, every four days, or every eleven days. In general, the activities of the antibiotics tested were not appreciably influenced by the treatment schedule. The methylglyoxalbisguanylhydrazones were more effective when given daily than when given every 4 days or as a single treatment.

Combination therapy with amethopterin and Cytoxan showed that, with appropriate use of the drugs, treatment with the combination was superior to treatment with either drug alone. Urethane, which was inactive in itself, did not potentiate the activity of amethopterin, nitrogen mustard, or Cytoxan. 6-Azauracil, also inactive when employed along, did not potentiate the activity of 6-mercaptopurine.

Increasing emphasis has been placed on studies of the effectiveness of chemical compounds on drug resistance. To date, these have included antipurine- as well as antifolic-resistant

variants of L1210. Drug-resistant tumors are being used to uncover active compounds which may have clinical usefulness in cases which have become refractory to conventional treatment and to explore possible mechanisms of drug action.

The sarcoma 37 assay system:

A total of 70 compounds has been evaluated against early sarcoma 37 for ability to increase the life span of the mice. This tumor has been particularly insensitive to treatment and has therefore not been used routinely in the advanced form. Of the compounds tested against early S-37, alanine nitrogen mustard, glycine nitrogen mustard, Cytoxan, N-methylformamide and p-N,N-Bis(2-iodoethyl)aminobenzylphosphonic acid ethyl ester have provided appreciable increases in survival time. This tumor is of particular interest, because of the apparent lack of correlation between the degree of tumor inhibition and the increase in survival time attained as a result of therapy. For example, 6-mercaptopurine, Bayer 3231, and Actinomycin D were able to inhibit local tumor growth in mice with Sarcoma 37 but provided little, if any, increase in survival time.

Special studies employing sarcoma 37 have been directed mainly to investigations of the influence of the treatment schedule on the effectiveness of compounds and to the influence of caloric restriction on local tumor growth and survival time.

The Adenocarcinoma 755 assay system:

Unlike sarcoma 37, Adenocarcinoma 755 (Ca-755) has been quite sensitive to certain compounds, particularly the purine antagonists. In earlier studies in this program, 45 compounds were tested against early Ca-755. Extensive increases in survival time as well as tumor-free survivors were observed as a result of therapy with 6-mercaptopurine and some of its derivatives. The high degree of sensitivity of this tumor to therapy indicated that it could be employed more advantageously in an advanced form for the quantitative evaluation of drugs. Within the past year, 40 compounds have been tested for their ability to increase the survival time of mice with advanced Ca-755. Many purine derivatives elicited substantial increases, but none appeared to be more effective than 6-mercaptopurine. Among non-purines, Cytoxan was the only compound which showed activity in the same range as 6-mercaptopurine. Moderate activity was observed for Actinomycin D and 4-aminopyrazolo (3,4-d)pyrimidine.

Like Sarcoma 37, Ca-755 displayed no correlation between local tumor inhibition and survival time extension. For example, three pyrazolo(3,4-d)pyrimidines tested produced approximately 50% inhibition of the local tumor of early Ca-755 without providing substantial increases in survival time.

The Ehrlich ascitic tumor assay system:

In preliminary studies, eight compounds were tested for their ability to increase the survival time of mice with the Ehrlich ascites tumor. Of the compounds examined in the preliminary studies using this assay system, only N-methylformamide provided an appreciable increase in survival time.

The studies conducted under this program have been directed to (a) the selection of drugs for clinical trial, (b) the obtaining of basic information concerning the pharmacologic and biochemical activity of such drugs and (c) further animal experimentation of specific problems referred back from the clinic. Structure-activity studies among compounds in animal tumor assay systems are emphasized. The inclusion of additional assay systems, such as with virus-induced tumors, carcinogen-induced tumors, and spontaneous tumors, is anticipated.

8. New Antileukemic Agents

(a) Reduced derivatives of antifolics (with Schrecker, Mead, Venditti; Kisliuk and Friedkin of Tufts University Medical School): Catalytic hydrogenation of MTX and DCM, carried out by Drs. Schrecker, Mead and Kisliuk, has provided the new compounds tetrahydroamethopterin (THMTX) and tetrahydrodichloroamethopterin (THDCM), the latter in analytically pure form. Formylation of THDCM has yielded 5-formyl-THDCM, an analog of citrovorum factor. A study was made of the antileukemic effect of these compounds in mice inoculated with L1210. It was found that THMTX was somewhat less effective than MTX, and that its toxicity was greater than that of MTX. THDCM was not quite as effective as DCM, but superior to MTX, in that with treatment of advanced leukemia, survival times of 30-40 days were obtained over a wide dose range. Toxicity of THDCM was intermediate between that of MTX and DCM.

Citrovorum factor was shown to effectively reverse the antileukemic activity of THDCM, but was less effective than against an equimolar dose of DCM. In another study, citrovorum factor was shown to effectively protect against the toxicity

of THMTX. 5-Formyl-THDCM was non-toxic and had no antileukemic effect.

THMTX and THDCM were found to be inhibitors of dihydrofolic reductase, but only at a much higher concentration than their non-reduced parent compounds. Analogously, THMTX inhibited formate-C^{14} incorporation into adenine to a lesser extent than did MTX. Since, however, the reduced compounds are effective against L1210 leukemia at doses much lower than their parent compounds, it may be that their action is directed primarily against enzyme reactions in which reduced folic coenzymes participate. Further studies on these aspects are in progress.

(b) Fluorinated derivatives of amethopterin (with Venditti, Mead, Schrecker): 3'-Fluoroamethopterin and 3',5'-difluoro-amethopterin (DFM) were obtained from the Lederle Laboratories and tested against L1210. The former had an effect comparable to that of MTX. DFM showed activity comparable to that of DCM. This would indicate that the improved therapeutic effectiveness of DCM over MTX in L1210 leukemia is not due to steric hindrance of the halogen atoms, as believed previously, but to electronic interaction of the electronegative halogen atoms. To test this hypothesis, it is planned to synthesize and test 3',5'-dimethylamethopterin, in which the steric, but not the electronic effect is present.

9. Biochemical Mechanism of Action of Folic Acid Antagonists

(a) Correlation of purine biosynthesis and therapy with antifolics (with Schrecker, Mead): The effect of daily treatment with MTX and DCM on formate incorporation in vivo into the acid-soluble adenine of normal and leukemic mouse spleen was investigated. Doses were selected that produced optimal survival times of leukemic mice (75 mg/kg of DCM; 0.8 mg/kg of MTX). With DCM, a cumulative inhibitory effect on labelling of acid-soluble adenine was observed both in normal and leukemic spleen. This treatment also reduced spleen infiltration with leukemic cells. With MTX, however, a cumulative inhibitory effect was observed in normal, but not in leukemic spleen. The leukemic spleens continued to grow in size despite treatment. These findings are consistent with the fact that DCM is a more effective drug than MTX in the treatment of L1210.

(b) In vivo study of effect of folic acid coenzymes on action of antifolic agents (with Schrecker, Mead, Venditti):

Previous studies in our Section have shown that the toxicity and antileukemic effect of folic acid antagonists can be reversed by citrovorum factor (CF), both with single and daily treatment. Folic acid is effective only with administration of a single dose of antifolic agent. It is known that the antifolics, by inhibiting folic reductase, prevent the reduction of folic acid to tetrahydrofolic acid (THFA). The active coenzymes (10-formyl-THFA and 5,10-methenyl-THFA) can be formed either from THFA or from CF. This would explain why CF can nullify the effect of antifolics when folic acid is unable to do so. A study has now been made of the effect of THFA and 10-formyl-THFA on leukemic mice treated daily with MTX. Complete reversal of the antileukemic effect of MTX was obtained by concomitant treatment with either compound. 10-Formyl-THFA seemed somewhat more effective a reversing agent than CF; however, further studies are needed to settle this point. Dihydrofolic acid (DHFA) was nearly as effective a reversing agent as THFA. This was somewhat surprising since MTX inhibits the reduction of DHFA to THFA very effectively. Moreover, it has been claimed that the enzymes which reduce folic acid and DHFA to THFA are identical. Further detailed study in vivo and in vitro will be needed to explain this unforeseen finding.

(c) Biochemical studies of resistance to antifolics (with Schrecker, Mead, Friedkin, Humphreys): Two antifolic-resistant sublines of L1210 had previously been developed in our Section. One of them (C82) was obtained by serial transplantation of spleen from leukemic mice that were treated for several generations with MTX, then for several generations with DCM. The other (M66) was obtained by treating the sensitive variant L1210 for several generations with DCM, omitting the preliminary treatment with MTX. In collaboration with Dr. Friedkin, of Tufts University, it was found that both the local tumor and the spleen of the M66 variant had the same amount of dihydrofolic reductase activity as the sensitive L1210 leukemia, while in the C82 subline the level of dihydrofolic reductase was increased 30-fold. Formate-C^{14} incorporation into the acid-soluble adenine of spleen and local tumor was inhibited by antifolics to a much lesser extent in the C82 variant than in the sensitive L1210 line. With single treatment with MTX or DCM, some inhibition was still obtained in the C82 line. With daily treatment, however, no inhibition at all was observed after several days of treatment. This would suggest that there are still some sensitive cells present in the C82 antifolic-resistant line and that they are eliminated early in treatment. It tends to support the hypothesis that, in the C82 subline,

there is a mixed population of cells with varying amounts of dihydrofolic reductase, the larger proportion of cells having higher amounts of the enzyme. The cells that do not possess high activity may be the ones eliminated during the first few days of treatment. Whether in the original L1210 line some cells with high dihydrofolic reductase activity are already present, has not yet been determined. In the M66 line, inhibition of formate incorporation by MTX or DCM (single treatment) is of the same order as in the sensitive line. At present the effect of multiple treatment in the M66 line is under study. Preliminary results indicate that, here again, treatment induces drug tolerance. It would appear, so far, that the mechanism of resistance may be different in the M66 variant from that in the C82 variant. In the latter a high enzyme activity produces a high tolerance for the inhibitor.

(d) Duration of effects of antifolic agents on dihydrofolic reductase and purine biosynthesis (with Schrecker, Mead, Friedkin): A comparison has been made of the effect of DCM and MTX on formate-C^{14} incorporation in vivo into the acid-soluble adenine of leukemic mouse spleen and on dihydrofolic reductase activity in the same tissue. Essentially complete inhibition of both formate incorporation and enzyme activity was found 20 minutes after injection of DCM or MTX. Recovery from inhibition was more rapid with formate incorporation than with dihydrofolic reductase. This difference appears to indicate that inhibition of dihydrofolic (and folic) reductase is not the only cause for inhibition of purine nucleotide biosynthesis. Our results would seem to indicate that, with the relatively high concentrations of antifolic present originally, enzymes other than folic reductase are also inhibited, and that, as the plasma and tissue concentration of antifolic decreases, only dihydrofolic (and folic) reductase remains inhibited.

(e) Clinical folic reductase studies (with Schrecker, Frei, Friedkin): Preliminary studies were made on dihydrofolic reductase in human leukemic cells. So far, reproducible results have not been obtained, although relatively low levels of the enzyme were observed in one patient. It appears that the enzyme is more readily denatured in human leukocytes than in the animal tissues studied previously. Thus, acetone fractionation, which is possible with animal liver and L1210 tumor, denatured the enzyme from the leukocytes.

(f) The persistence of amethopterin in mammalian tissues

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82

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(with Condit, Charache, Humphreys): Amethopterin persisted in the tissues of man and mouse for more than 100 days after the cessation of drug administration.

The amount of drug remaining and the duration of its persistence were not clearly related to the size of the administered dose or to the duration of its effects.

Protection against the lethal toxicity of amethopterin by administration of citrovorum factor did not prevent fixation of amethopterin in the tissues.

10. Pyridine Precursors of Diphosphopyridine Nucleotide (DPN) (with Humphreys, Ciotti, Kaplan): The fact that injection of nicotinamide in the mouse resulted in more than a 10-fold increase in liver DPN led to an investigation of pyridine compounds as possible coenzyme precursors. We had determined that injection of pyridines such as 3-acetylpyridine, pyridyl-3-methyl carbinol, β-picoline, monomethyl and monoethyl nicotinamide also result in rapid formation of liver DPN.

It had, in addition, been determined that pyridine derivatives may not only serve as precursors of DPN, but may also form analogs DPN in vitro and in vivo by an exchange reaction involving DPNase. The analogue of acetylpyridine, for example, has been found in the leukemic tissue of mice following injection of this compound.

Structure-activity studies were undertaken on the toxicological and biochemical interrelationships with the pyridine derivatives. Dose response curves have now been obtained for approximately 50 pyridine derivatives. These data will not be detailed here except to indicate that within structural series, substitutions may produce marked alterations in toxicity. The relatively non-toxic compounds have been tested for their ability to protect, as does nicotinamide, against the lethal toxicity of β-acetylpyridine and various others of the toxic derivatives. For example, N-monomethyl nicotinamide, nicotinhydroxamic acid and diphosphopyridine nucleotide can replace nicotinamide in protecting against β-acetylpyridine toxicity. N-monoethyl nicotinamide gives rise to increased liver DPN on injection, as does nicotinhydroxamic acid. Notably, no protection was afforded by nicotinic acid. The latter may actually inhibit DPN synthesis from nicotinamide.

Compounds such as pyridyl-3-methyl carbinol, 6-aminonicotinamide, and several thiadiazole derivatives resemble β-acetyl

pyridine in that nicotinamide and compounds with nicotinamide activity afford protection against toxic doses. Nicotinamide has also been shown to reverse the antileukemic action of 2-amino-1,3,4-thiadiazole and 2-ethylamino-1,3,4-thiadiazole. Pyridyl-3-methyl carbinol will not only form analog of DPN, but also gives rise to increased DPN on injection.

A number of the pyridine compounds form analogues of DPN and/or give increased DPN synthesis, but behave neither as nicotinamide antagonists nor as nicotinamide on injection.

Of the compounds in this series, tested to date, only 6-aminonicotinamide, β-acetylpyridine and the thiadiazoles have evidenced any antileukemic activity.

Parallel in vivo pharmacological and biochemical studies may help to elucidate further the role of pyridine coenzymes. More adequate control of host toxicity may lead to enhanced antitumor action.

11. Lactic dehydrogenase (with Kaplan, Humphreys, Schrecker): Studies on the influence of antileukemic agents on lactic dehydrogenase activity in leukemic blood (as well as tissues) are in progress. A sharp rise was found in lactic dehydrogenase activity of blood in advanced disease. In preliminary studies, antifolic therapy was shown to result in diminished activity. It is of interest, as shown by Kaplan, that analogues of DPN such as acetylpyridine-DPN can inhibit lactic dehydrogenase activity viz. the reduction of pyruvate by DPNH. The reduced acetylpyridine analog, although acting as an inhibitor of lactic dehydrogenase with DPNH as the reactant, may itself react at a slow rate with pyruvate. Additional knowledge of analog formation and metabolic participation may be useful in studies of inhibition of lactic dehydrogenase and other enzyme systems by chemotherapeutic agents.

12. Phenylalanine Mustard (Sarcolysin) Assay (with Chirigos): Phenylalanine mustard, 3-(p-/Bis(2-chloroethyl)amino/phenyl-DL-alanine, has demonstrated chemotherapeutic activity against a variety of tumors. The growing interest in this antitumor agent has indicated the need of methods for its determination. A simple fluorometric method has now been developed which is rapid, sensitive, and precise for measuring small quantities of phenylalanine mustard in tissues.

Measurement of the drug in vivo involves removal of tissues from animals previously injected with the drug, appropriate

extraction, and measurement of native fluorescence in the Aminco-Bowman Spectrophotofluorometer.

Following administration of a therapeutic dose of the drug (SC), the highest concentration in plasma occurred at 1 hour, and in the kidney at 2 hours. Following IP administration, the highest concentration of drug occurred in plasma in 15 minutes, and in Sarcoma 37 in 1 hour.

In a preliminary study, a comparison was made of recovery of phenylalanine mustard following incubation with heparinized rat whole blood and plasma. Following incubation in plasma, 90% recovery of Sarcolysin occurred at 5, 15, 30, and 45 minutes. In contrast, recovery from whole blood was less; at 15, 30, and 45 minutes respectively, only 88, 74, and 64% recovery of phenylalanine mustard occurred. The results indicate that cellular fractions of whole blood may be binding phenylalanine mustard.

The phenylalanine mustard assay will be employed in studies of transport of the L-, D-, and DL- enantiomorphs of phenylalanine mustard in leukemia, ascites tumors systems, etc., and as an additional tool in study of mode of action.

13. Biochemical Studies of Tumor Transamination (with Chirigos): Transaminase activity was surveyed in a series of transplantable and spontaneous tumors.

The ability of cell-free extracts to mediate the transfer of the α-amino groups to parahydroxyphenylpyruvate with the formation of tyrosine when any of 7 different -amino acids was added to the extracts was tested in eight transplanted tumors, and in eight spontaneous tumors, as well as in livers from four different strains of mice.

Phenylalanine, alanine, cysteine, methionine, glutamic acid, glutamine, and aspartic acid were observed to be active amino donors in all of the liver extracts.

A lesser degree of similarity in transaminating pattern was observed in tumors as compared with liver. Tumor extracts demonstrated greater transaminating activity with glutamic acid than with glutamine, in contrast to liver preparations, which exhibited similar activities with both amino acids. The role of glutaminase has so far not been estimated in the various tissues.

Liver extracts from tumor-bearing mice exhibited transaminating activity similar to that observed in normal mouse liver extracts.

Several experiments were conducted to ascertain whether the transamination reactions observed in tumor and liver extracts were mediated by known transaminating enzymes. The results indicate that the observed transamination reactions in tumor and liver extracts are attributable to an enzyme or enzymes which have not been reported to date. A partial fractionation of this enzyme(s) has been completed. Further investigations are presently being conducted to identify the enzyme(s).

14. Histochemistry (with Chirigos, Glenner): A collaborative research program is in progress with Dr. George Glenner, of the Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases, in which the cellular localization and concentration of tyrosine and tryptophan in the mammary Adenocarcinoma 755 is being studied.

If the method proves applicable it will be utilized to study the following: (1) The tyrosine and tryptophan content of tumors responding to effective chemotherapy; (2) Uptake of the amino acids by tumor tissue when injected into the host; (3) The comparative cellular localization and concentration of these amino acids in a variety of tumors, including spontaneous tumors, and the response to chemotherapy.

15. Antileukemic Action of Reserpine (with Salvador, Burton, Humphreys): Shore and Brodie (NHI) showed that, following pretreatment of rabbits with iproniazid (a monoamine oxidase inhibitor), reserpine administration resulted in excitatory and sympathomimetic responses. In an effort to dissociate the central depression induced by reserpine from its antitumor activity, experiments were conducted to determine the effect of prior administration of iproniazid on the antileukemic effectiveness of reserpine in mice.

Iproniazid was capable of reversing the action of reserpine against advanced L1210. It reduced the median survival time to that of the controls over a wide dose range of reserpine. Iproniazid alone did not alter the rate of growth of the leukemia. The activity of 3-isoreserpine, a stereoisomer of reserpine, was of particular interest, since this compound is known to be devoid of central nervous system depressant effects. The combination of 3-isoreserpine and reserpine gave additional survival time of leukemic mice over that observed with reserpine

alone, suggesting a potentiating efffct by isoreserpine. This dose of isoreserpine alone was ineffective.

Iproniazid appeared to reverse the depressant action both of reserpine and of reserpine in combination with 3-isoreserpine. It also diminished the loss of body weight and inhibition of local tumor growth. Thus, the experiments failed to demonstrate a dissociation of the central nervous system depressant action of reserpine from the ability of reserpine to inhibit leukemic growth. Preliminary studies have indicated that reserpenized mice, when aroused with iproniazid, have diminished food intake. It still remains to be determined whether the above correlation is indicative of a nonspecific host mediated antileukemic effect or whether there is a specific antitumor action of reserpine.

16. Interaction of Nicotinamide with Reserpine and Chlorpromazine (with Burton, Kaplan, Humphreys): Although massive doses of nicotinamide increase the DPN level of animal tissues, these elevated levels quickly return to the basal level unless the animal has been treated with a chemical agent such as reserpine or chlorpromazine. A correlation has been obtained between the ability of reserpine and reserpine derivatives and chlorpromazine and phenothiazine derivatives to tranquilize animals and their ability to maintain elevated DPN levels. Non-tranquillizing sedatives, such as phenobarbital and ethanol are not able to maintain these high DPN levels. Evidence has been presented which indicates that this action of the tranquillizing agents is not mediated through serotonin.

Significance to the Program of the Cancer Institute:

These studies are designed to provide basic pharmacological and biochemical information on host-tumor interrelationships in chemotherapy. From such studies, principles for treatment in animals are being established which may be of value in application to clinical chemotherapy. In addition to fundamental biochemical and pharmacological studies, methodology is provided by the program for the evaluation and development as well as investigation, of compounds of potential clinical interest.

Proposed Course of Project:

During the next year the various phases of this work will be continued and broadened. Investigations involving quantitative evaluation of antitumor effectiveness will include:

structure-activity studies, inhibition analysis (synergism and antagonism), and projects dealing with sublines of tumors resistant to antitumor agents. Studies will be expanded with virus leukemia, including chemotherapy of the induced disease, transplanted disease, and the virus proper. Projects in progress with spontaneous tumors will be continued. The use of carcinogen induced tumors, including lung tumors, as tools in experimental chemotherapy will be explored.

Studies of the interrelationships of chemotherapy and immunity and chemotherapy and surgery will be continued.

Fate and distribution studies will be continued, including further application to intracranial tumors.

Investigations of biochemical mechanisms will be expanded. This area includes host-tumor studies of the influence of chemotherapeutic agents on biosynthesis, including purine synthesis, etc.; in vivo and in vitro studies of enzyme systems (including clinical material); mechanisms of resistance; transamination; in vivo analog formation; lactic dehydrogenase studies, etc.

Various areas of investigations with other intramural groups and extramural institutions will be continued.

Part B included: Yes

PHS-NIH
Individual Project Report
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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Goldin, A., Venditti, J. M., Humphreys, S. R., Shuster, L., Darrow, R. A., and Mantel, N.: Advanced Leukemia as a Tool for Chemotherapeutic Studies. ACTA UNIO INTERNATIONALIS CONTRA CANCRUM XVI, 642-650, 1960.

(2) Goldin, A., and Humphreys, S. R.: Studies of Immunity in Mice Surviving Systemic Leukemia L1210. J. Nat. Cancer Inst. 24, 283-300, 1960.

(3) Goldin, A., Humphreys, S. R., Chapman, G. O., Chirigos, M. A., and Venditti, J. M.: Immunity of Mice Surviving Systemic Leukemia (L1210) to Antifolic Resistant Variants of the Disease. Nature 185, 219-221, 1960.

(4) Charache, S., Condit, P. T., and Humphreys, S. R.: Studies of the Folic Acid Vitamins. IV. The Persistence of Amethopterin in Mammalian Tissues. Cancer 13, 236-240, 1960.

(5) Charache, S., Condit, P. T., Levy, A. H., Humphreys, S. R., and Goldin, A.: Studies on the Folic Acid Vitamins. V. Effect of Folic Acid on the Citrovorum Factor Activity of Mouse Liver. Cancer 13, 241-244, 1960.

(6) Venditti, J. M., Frei, E. III, and Goldin, A.: Effectiveness of Pyrazolo(3,4-d)Pyrimidines Against Transplantable Mouse Tumors. Cancer 13, 959-966, 1960.

(7) Burton, R. M., Salvador, R. A., Goldin, A., and Humphreys, S. R.: Interaction of Nicotinamide with Reserpine and Chlorpromazine. II. Some Effects on the Central Nervous System of the Mouse. Archives Internationales de Pharmocodynamics et de Therapie. In press.

(8) Burton, R. M., Kaplan, N. O., Goldin, A., Leitenberg, M., and Humphreys, S. R.: Interaction of Nicotinamide with Reserpine and Chlorpromazine. III. Some Effects on the Synthesis of Diphosphopyridine Nucleotide in Liver. Archives Internationales de Pharmocodynamics et de Therapie. In press.

(9) Darrow, R. A., Shuster, L., and Goldin, A.: Amethopterin and Liver Purine Metabolism. Cancer Research 20, 620-627, 1960.

(10) Ciotti, M. M., Humphreys, S. R., Venditti, J. M., Kaplan, N. O., and Goldin, A.: The Antileukemic Action of Two Thiadiazole Derivatives. Cancer Research 20, 1195-1201, 1960.

(11) Goldin, A., Humphreys, S. R., Chapman, G. O., Venditti, J. M., and Chirigos, M. A.: Augmentation of Therapeutic Efficacy of 3',5'-Dichloroamethopterin Against an Antifolic Resistant Variant of Leukemia (L1210-M46R) in Mice. Cancer Research 20, 1066-1071, 1960.

(12) Venditti, J. M., Goldin, A., and Kline, I.: The Influence of Treatment Schedule on the Chemotherapy of Advanced Leukemia L1210 in Mice. Cancer Chemotherapy Reports 6: 55-57, 1960.

(13) Schrecker, A. W., Mead, J.A.R., Lynch, M. R., and Goldin, A.: Comparative Effect of Amethopterin and its 3',5'-Dichloro Derivative on Purine Biosynthesis in vivo in Leukemic Mice. Cancer Research 20, 876-886, 1960.

(14) Chirigos, M. A., Humphreys, S. R., and Goldin, A.: Intracerebral Growth and Treatment of Leukemia L1210. Proc. Soc. Exp. Biol. & Med. 104, 643-646, 1960.

(15) Venditti, J. M., Frei, E. III, and Goldin, A.: The Effectiveness of 2-Amino-6-(1'-Methyl-4'-Nitro-5'-Imidazolyl) Mercaptopurine Against Transplantable Mouse Leukemia. Cancer Chemotherapy Reports 8, 44-46, 1960.

(16) Goldin, A., Venditti, J. M., Kline, I., and Mantel, N.: Evaluation of Antileukemic Agents Employing Advanced Leukemia in Mice. II. Cancer Research 20, Cancer Chemotherapy Screening Data VI, 382-448, 1960.

(17) Venditti, J. M., Humphreys, S. R., Mantel, N., Kline, I., and Goldin, A.: Evaluation of Antileukemic Agents Employing Advanced Leukemia L1210 in Mice. III. Congeners of Folic Acid. Cancer Research 20, Cancer Chemotherapy Screening Data VIII, 698-733, 1960.

(18) Venditti, J. M., Schrecker, A. W., Mead, J.A.R., Kline, I., and Goldin, A.: Influence of the Route of Administration on the Relative Effectiveness of 3',5'-Dichloroamethopterin and Amethopterin Against Advanced Leukemia (L1210) in Mice. Cancer Research 20, 1451-1456, 1960.

(19) Schrecker, A. W., Mead, J.A.R., Lynch, M. R., Venditti, J. M., and Goldin, A.: Effect of Orally Administered 3',5'-Dichloroamethopterin on Formate-C^{14} Incorporation in Leukemic Mice. Cancer Research 20, 1457-1461, 1960.

(20) Misra, D. K., Humphreys, S. R., Friedkin, M., Goldin, A., and Crawford, E. J.: Increased Dihydrofolate Reductase Activity as a Possible Basis of Drug Resistance in Leukemia. Nature. In press.

(21) Goldin, A., Venditti, J. M., Kline, I., and Mantel, N.: Evaluation of Antileukemic Agents Employing Advanced Leukemia L1210 in Mice. IV. Cancer Research, Cancer Chemotherapy Screening Data. In press.

Honors and Awards relating to this project:

A. Goldin: (a) Received reappointment as Visiting Professor of Biochemistry at Brandeis University, Waltham, Massachusetts; (b) Panel Member, Board of U. S. Civil Service Examiners at NIH; (c) Foreign Corresponding Member, Societa Italiana Di Cancerologia; (d) Affiliate membership, Royal Society of Medicine; (e) Member, Cancer Chemotherapy National Service Center Drug Evaluation Panel, and its Committee on Experimental Therapeutics; (f) PHS Member, Cancer Chemotherapy Study Section, Division of Research Grants; (g) Appointed Consultant in Research to Graduate Council, George Washington University School of Medicine; (h) Invited to Chair session on Chemotherapy, 51st Annual Meeting of American Association for Cancer Research; (i) Invited to present data at Review of Antifolic Research at NCI, February 23, 1960; (j) Invited to present data at, and participate in conference on Drug Development Program for Responsive Tumors in Man, August 8-9, 1960; (k) Invited to present work of Laboratory at Department of Zoology Colloquim, University of Maryland, March 23rd, 1960; (l) Invited to present data at CCNSC conference on Purines, October 4, 1960; (m) In collaboration with Clinical and Professional Education Branch, and jointly with General Medicine Branch, NCI, filmed work of the laboratory for use in Public Health Service Orientation programs, April 19, 1960.

A. Schrecker: (a) Elected to membership in American Association for Cancer Research, Inc.

J. Venditti:(a)Elected to membership in American Association for Cancer Research, Inc. (b) Invited to present data at, and participate in, conference on Drug Development Program for Responsive Tumors in Man, August 8-9, 1960.

Serial No. NCI-252
1. Lab. Chemical Pharmacology
2. Cellular Pharmacology Section
3. Bethesda 14, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Pharmacologic Action of Tumor-Damaging and Other Chemical Agents on Animal and Human Malignant and Normal Cells.

Principal Investigator: Dr. M. Belkin.

Other Investigators: H. C. Orr, A. Perrault.

Cooperating Units: Dr. A. J. Dalton, Cellular Biology Section, Serial No. NCI-406; Dr. J. Preiss, NIAMD-LPB-3; Dr. D. Lavie, Weizmann Institute, Rehovoth, Israel.

Man Years (Calendar Year 1960)		Patient Days
Total:	6	None
Professional:	2	
Other:	4	

Project Description:

Objectives:

a) To study tumor necrotizing compounds, from plant and other sources, to determine how they bring about cell damage and cell death; b) to study the morphologic and biochemical responses of normal and malignant cells to various drugs and chemical agents with particular regard to certain basic structures and functions of the cell, i.e., the behavior of the cellular membrane complex, permeability to water and salts, enzyme function, etc.

Methods Employed:

Classical techniques of pharmacology, cyto- and histochemistry, and tissue culture are used. New techniques are developed as necessary.

Major Findings:

A. Studies in Cellular Pharmacology

I. Factors Regulating Control of Cell Permeability to Water and Salts.

In the previous Annual Report, there was described the induction of 3 types of blebs in ascites tumor cells which were subjected to certain chemical agents. The forms of these blebs were described as (1) acentric, (2) symmetrical and (3) scallop (numerous small blebs which surround the cell).

Many chemical agents have been tested for ability to produce blebs. All those capable of doing so have the property of reacting with protein-sulfhydryl groups by alkylation, oxidation or mercaptide formation.

To date, the 3 bleb types have been induced in eight mouse and two rat ascites tumor cells; in four human and one mouse malignant cell line plus three normal cell lines grown in tissue culture. In contrast, cells from normal solid tissues of liver, lung, spleen, kidney, testis and brain from mouse, rat and rabbit failed to produce blebs.

The following interpretations are presented for the above findings:

1) Bleb formation is associated with the interruption of the role played by sulfhydryl groups in maintaining the structural and functional integrity of the plasma membrane and thereby its normal permeability. This suggestion is supported by many observations which link inactivation of —SH groups to a variety of structural and functional changes.

2) The capacity to bleb may be related to the malignant state of the cell or to the manner in which the cell has been maintained, i.e., every blebbing cell has been a malignant cell, or a normal cell grown in tissue culture as a free or unattached cell and not as part of a solid aggregate.

Hence the questions may be raised 1) whether a cell in process of converting to the malignant state or adapting to independent growth in a new environment to survive must undergo certain structural and functional changes with respect to permeability and other properties and 2) whether such changes are

reflected in its increased sensitivity to the action of a blebbing agent. The capacity to bleb readily may be a criterion of any malignant cell, or of a normal cell which has acquired the ability to exist independently.

These observations have been embodied in a manuscript which is in press.

B. The Induction of Intracytoplasmic Vacuoles in Ascites Tumor Cells.

I. In vivo induction of cell vacuoles.

In a previous report (Belkin, et al, Cancer Res. 19: 1050-62, 1959) there has been described the production in vivo of cytoplasmic vacuoles following the intraperitoneal administration of plant polymers into mice carrying various ascites tumors. This phenomenon, which can be elicited only in vivo, appears to be the result of an antigen-antibody reaction, a substance in the plant material acting as antigen.

This concept is supported by experiments in which ascites tumor cells were first exposed to a preparation from goldenrod polysaccharide, then thoroughly washed, and placed in antiserum from rabbits made immune to the polysaccharide fraction. The cells appeared damaged, and gave evidence of an antigen-antibody reaction at the cell surface.

To date, the origin of these vacuoles in the cells of 3 ascites tumors has been examined with the electron microscope (in collaboration with Dr. A. J. Dalton), and the process of vacuole formation in each case has been found to differ in certain respects.

1) In sarcoma 37 cells the vacuoles appear to arise primarily at the site of virus particle formation; but others arise also from preformed small vacuoles and "inclusion bodies" which are occasionally present.

2) Vacuoles develop in Yoshida ascites cells from preformed small vacuoles and membrane-enclosed, electron-dense inclusion bodies.

3) In Ehrlich ascites cells, few preformed vacuoles are present in untreated cells, and the vacuoles develop more slowly from membrane-bound, electron-dense "inclusion bodies"; the cells of this variant of Ehrlich ascites tumor were rarely found to contain virus particles.

It is of interest that in the 3 tumors examined thus far, the cell components primarily concerned with vacuole formation are specialized membranes involved in virus particle formation, preformed small vacuoles and membrane-bound electron-dense "inclusion bodies", rather than typical cytoplasmic organelles such as the mitochondria, or the Golgi complex.

Continued study of several other tumors will reveal whether these findings will be general for all tumors examined.

II. In vitro induction of cell vacuoles.

The vacuolar phenomenon described above has been elicited only in vivo following the parenteral injection of plant polymers. However, the same response can also be obtained in ascites tumor cells when they are exposed in vitro to various autonomic drugs.

To date, 35 autonomic drugs, representative of all types of such compounds acting on the central and peripheral nervous system have been tested. Of these, fifteen compounds distributed among all autonomic groups have been found to induce intracytoplasmic vacuoles in cells of four different ascites tumors: sarcoma 37, Ehrlich carcinoma, hepatoma 134 and leukemia L1210.

These vacuoles appear microscopically identical with those resulting from polysaccharide administered in vivo. The vacuoles may at times be discernible as early as one hour after drug exposure; but usually it takes 2-3 hours for the phenomenon to become full blown.

To date, the normal cells of liver, kidney, spleen and testis have not produced vacuoles when exposed to six agents arbitrarily selected from among the many autonomic drugs active against ascites tumor cells.

The origin of these vacuoles is at present unknown. It is anticipated that electron microscope studies will help to clarify the question of their mechanism of formation.

It is of interest that the plasma membrane involved in bleb formation and the substance forming the vacuolar walls, both belong to the cellular membrane complex. During the past decade, such membrane structures which also include the endoplasmic

reticulum and the mitochondrial membranes have been shown increasingly to have importance in the tectonic and functional economy of the cell. Whether vacuoles are internal blebs and blebs are external vacuoles is at present a semantic quibble. An intimate relationship may be disclosed as a result of further study; however, in the experiments reported here they were each induced by different classes of chemical agents indicating certain dissimilarities.

C. Evaluation of Vital Stains as Indicators of Cell Injury and Death.

This investigation is concerned with the validity of vital stains as indicators of viability. Employing locomotion in the ciliates Parameciun and Colpidium as a criterion of viability, the behavior of several vital dyes has been investigated as previously described.

Another motile protozoan, Ameba proteus, has been tested in addition to the ciliated organisms. In general the behavior of vital dyes as indicators of injury in amebas closely parallel the results obtained with the ciliates. One interesting difference observed that that the phenomenon of metachromasia with Trypan blue was exhibited by the ameba and not by the ciliates, whereas the reverse behavior was obtained with Toluidine blue.

D. Studies with Human Malignant Cells in Tissue Culture.

When cells growing in tissue culture such as those of strain HeLa, D189 and others are dried over phosphorus pentoxide for 30-60 minutes, a considerable amount of the architectural integrity of the cell is preserved. When such cells are "reconstituted" by adding water to them, their morphology is surprisingly good as observed with phase contrast microscopy and compared with untreated cells.

Such dehydrated cells, furthermore, display the capacity to stain for certain cytologic constituents. Typical staining is obtained for fat granules with Oil-Red-O, and for polysaccharides with periodic acid-Schiff reagent. However, cell components like chromosomes, proteins and nucleic acids when stained with hematoxylin and eosin or iron-hematoxylin do not appear as well stained following dehydration as they do after conventional chemical fixation.

The process of physical fixation by rapid dehydration is being explored to determine its usefulness. Its advantages as a fixing agent are (1) simplicity, (2) rapidity, (3) permits storage for indefinite periods, allowing decisions to be made later with regard to further treatment, (4) certain cytologic constituents appear less damaged following dehydration than after chemical fixation.

Fixation in this manner by non-chemical means may avoid chemical changes induced by routine chemical fixatives, and may prove to be of value to the cyto- and histo-chemist.

E. Electron Inactivation of Plant Polysaccharide.

In last year's report, experiments were described in which goldenrod polymer was bombarded in vacuo with 7 K.V. electrons emitted by a hot tungsten filament. Although polysaccharide controls always induced typical vacuolization in sarcoma 37 ascites tumor cells, the treated samples displayed progressive inactivation with increasing electron dose, till 98% inhibition was obtained.

The value of the molecular weight of the active moiety of the polymer was found to be about 9000, and was obtained by comparing its inactivation function with that of two globular proteins, ribonuclease and invertase, which were subjected to the same treatment. The size of the active portion of what is undoubtedly a large molecule is much smaller than anticipated.

Further work should disclose whether other properties of the polymer have been attenuated or altered by electron exposure, i.e., pyrogenicity, resistance to subsequent injections, capacity to lower blood pressure, etc.

Significance to the Program of the Cancer Institute:

These studies are designed to obtain basic knowledge with regard to (1) morphological and functional differences between malignant and normal cells, (2) to correlate at the cellular level the pharmacologic action of drugs with the structural and functional responses of cells in relation to treatment, (3) the mechanisms by which specific necrotizing compounds produce cellular damage. Such information is fundamental to a better understanding of drug action; and to help in the development of newer and better drugs for use in clinical chemotherapy.

Proposed Course of Project:

The major problems of this project will be continued, with specific attention to (1) studies of the factors regulating water and salt permeability and its relation to structural and functional changes in the plasma membrane, (2) the nature of cell damage due to chemical agents, (3) the development of improved methods to recognize such damage.

Part B included: Yes

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Orr, H. C., and Belkin, M. Characterization by Certain Drugs of Four Morphologically Indistinguishable Cell Lines. J. National Cancer Institute 25: 813-819, 1960.

(2) Vivian, D. L., Hogart, R. H., and Belkin, M. Phenazine Syntheses. X. 2,8-Disubstituted Phenazines Made as Intermediates for New Vital Stains, together with Two New Vital Stains Related to Neutral Red. J. Organic Chemistry, Vol. 25, December 1960.

(3) Belkin, M., and Hardy, W. G. Relationship between Water Permeability and Integrity of Sulfhydryl Groups in Malignant and Normal Cells. J. Biophysical and Biochemical Cytology. In Press.

Honors and Awards pertaining to this project:

Elected to Membership in the Washington Academy of Sciences.

Serial No. NCI-262
1. Laboratory of Chemical Pharmacology
2. Macromolecular Chemistry Section
3. Bethesda 14, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Study of macromolecular interaction to alter activities of enzymes, viruses, and other biological systems.

Principal Investigator: P. T. Mora

Other Investigators: P. Maury, S. I. Oroszlan, S. Rizvi, S. Shifrin, J. W. Wood, and B. G. Young.

Cooperating Units: Dr. Philip Person, Chief, Special Dental Research Laboratory, Special Dental Research Program, Veteran's Administration Hospital, Brooklyn, New York; Dr. A. Di Marco, Director of Research, Farmitalia, Professor of Pharmacology, University of Milan, and Instituto dei Tumori, Milan, Italy; Professor Rolf Meier, Ciba Ltd., Basle, Switzerland.

Man Years: (calendar year 1960)		Patient Days
Total:	10	None
Professional:	5	
Other:	5	

Project Description:

Objectives

Study of the interaction of biological systems on the macromolecular level, with special emphasis on the forces which initiate the interactions, and which orient the interacting biological systems to make the interaction efficient. We wanted to understand better how it is that the biological interactions seek the "effective" ways, and to what extent the biological interactions on the molecular level differ from the purely chemical interactions of nonbiological systems where there is no orientation or selection. The basic knowledge arrived at in these studies was then applied to inhibit (or to enhance) enzymes, to inhibit viruses such as bacteriophage, and to alter some of the biologic activities of a bacterial endotoxin.

Methods Employed

The methods were selected from the fields of biophysics, polymer chemistry, synthetic organic chemistry, etc. Such physical chemical methods as ultracentrifugation, electrophoresis, chromatography, polyelectrolyte titrations, viscometry, optical methods, etc. were employed. The data from these measurements were correlated with results from various in vitro and in vivo biological assays, such as enzyme assays, phage viability assays, toxicity determination, tumor necrosis and fever production in animals, etc. In the virus work, radio-active protein and nucleic acid virus components were extensively employed to study the mechanism of infection of bacteriophages.

Major Findings

I. Enzyme substrate interaction

There is a well-known common feature in the action of all of the enzymes: their interaction with their substrate is very efficient, as if every collision would bring about a reaction. This is different from an uncatalyzed chemical interaction of molecules.

We have found in our earlier work, that electrostatic forces operating between the enzyme and the substrate are important in bringing together these two molecules. We studied this by the inclusion of a third, synthetic, charged macromolecular component, and we learned something about the nature of the electrostatic attracting force between the enzyme and the substrate, by overcoming it with the third synthetic component which had the same kind of net charge as the substrate, but of higher magnitude. The utility of this third component resides in the fact that we can alter chemically its molecular weight and, what is most important, its charge. We employed chemically synthesized polysaccharide derivatives, substituted both with negative (acid)groups and with positive (basic) groups.

We found in earlier work with B. G. Young that, for example in the interaction of the positively-charged enzyme ribonuclease (RNase) with the negatively-charged substrate ribonucleic acid (RNA), we could inhibit the enzyme by including a more negatively-charged macromolecule than RNA, such as a polyglucose sulfate or a polyglucose carboxyl derivative. The amount and the acidity (pKa) of the disassociating groups on the synthetic polysaccharide derivatives were the most important controlling parameters of the inhibition. There was complete inhibition of 10 µg. RNase with 10 µg. polyglucose sulfate or with 20 µg. polyglucose carboxyl

if both synthetic derivatives had high-charge density, but the inhibition rapidly decreased when we decreased the charge density of the synthetic polysaccharide derivatives. Kinetic measurements of enzyme activity, using changes in the UV spectrum (Kunitz assay) were carried out at the pH optimum 4.7. We are now extending our studies to higher pH levels (6-7), closer to physiological conditions, using other assays of RNase; and we are also studying the effect of salt concentration on the inhibition. At pH 7 the ribonuclease is less positively charged, and the electrostatic forces are not so strong between the RNase and the polysaccharide derivatives: we observe inhibition, but larger amounts of negative polyelectrolytes are required.

Speculative conclusions from our studies are as follows (cf. ref. 2):

(1) Electrostatic forces apparently initiate the interaction between the substrate and the enzyme, and predominate in the early stages of the interaction. The required specific enzyme substrate complementariness comes into the reaction later, after the electrostatic forces (due to a just appropriate "built in" distribution of charge into the substrate and the enzyme) cause approach and interaction at the active sites. The two centers of opposite charges in the two molecules polarize the charge distribution in the enzyme during approach and in consequence, the enzyme and the substrate attract each other even more strongly, until finally the interaction happens at the complementary sites.

(2) The optimum pH and salt concentration which alter the electrostatic attracting forces is necessary to insure that the enzyme and the substrate will indeed interact with a rate and speed just optimum to allow development of interaction through the active centers. In other words, while the macromolecules are apart and have rotational freedom, there is enough time that the two molecules can turn around and face each other and collide at complementary sides.

In the course of our research a need arose to study the interaction of the enzymes and the inhibitors by electrophoresis, and by ultracentrifugation. We had difficulty, however, in developing these techniques to be able to observe the components at the low concentrations of our enzyme inhibition studies.

There is a highly sensitive method available to study macromolecular interaction: fluorescence spectrophotometry. Dr. Sidney Shifrin joined the Section as a new staff member to

work in this field. Dr. Shifrin, at Brandeis University, studied the binding of pyridine coenzymes, such as reduced diphosphopyridine nucleotide (DPNH), to dehydrogenase, such as horse liver alcohol dehydrogenase. Dr. Shifrin is engaged now in synthetic work, preparing certain specific chemicals, to study enzyme coenzyme and substrate interaction by the fluorescence technique. He is planning to construct a suitable fluorescence spectrophotometer for this study.

During our inhibition studies with B. G. Young, we observed not only inhibition, but in certain instances enhancement of enzyme activity. It appears that in tissues, or when the enzyme is not completely purified from the natural components occurring in the tissue, the enzyme may exist in a complex with a natural inhibitor, which may be removed by treatment with a polyelectrolyte. For example, following up on our general approach, Dr. Philip Person observed these phenomena: Cytochrome c oxidase can be effectively inhibited with a positive macrocation such as protamine but the activity can be restored completely by a negative macromolecule such as polyglucose sulfate. Adding somewhat more of the negative macromolecule, or by omitting the positive macromolecule from the system, a substantial increase in the cytochrome c oxidase activity can be obtained, probably by removing a naturally occurring positively-charged blocking component of the cytochrome c oxidase. We have observed similar phenomena on RNase activity (in presence of serum) and on DNase. Such leads are important in studying the natural balance of enzymes in tissues, especially since it is known that certain enzyme rates are different in neoplastic tissues from that in the normal tissues.

II. Interaction to block selective sites of biological macromolecules.

(a) Differentiation between enzymatic and antigenic sites. We began an attempt to differentiate these sites on a protein, such as RNase, by electrostatic complexing and blocking of certain limited portions of the protein surface. We found, however, that when we added unpurified RNase antibody to RNase, other serum components interferred with the enzymatic assay. Therefore, we decided to prepare purified RNase antibody by a new method (Singer). After the purified antibody is available we will see if such antibody would influence the enzymatic activity of the ribonuclease, and to what extent a negatively-charged macromolecule would inhibit the antigen-antibody interaction, or the in vivo development of antibodies to RNase.

(b) Differentiation of Active Sites on a Bacterial Endotoxin from Serratia marcescens. The new Serratia marcescens preparation P-45, was found to be effectively deaggregated by treatment with the anionic detergent: sodium lauryl sulfate. Sedimentation of the original P-45 in the ultracentrifuge shows two peaks, one $S^w_{20} = 4$, and another $S^w_{20} = 12$. After treatment with sodium lauryl sulfate there appears only one peak at $S^w_{20} = 2$. However, up to now, we could not remove the sodium lauryl sulfate without causing reaggregation. The biological activity of the $S^w_{20} = 2$ component was unchanged in the presence of the sodium lauryl sulfate. This indicates that the biological activity is retained in a relatively small molecular weight unit of about 100-200,000.

The S. marcescens endotoxin is an acid polysaccharide complex. In an earlier work with Dr. Merler we found 80-100 negatively-charged groups with pKa = 2.3 in a 100,000 molecular weight unit. With B. G. Young we studied the titration of the negative polysaccharide with positively-charged macromolecules, such as polymyxin, protamine, lysozyme, etc. There was a neutralization observed at a pH range where the maximum increase in turbidity indicated that strong macromolecular interaction (precipitation) took place. Generally, with the positive macromolecules the tumor necrotizing activity decreased, but the pyrogenic activity remained constant. However, after interaction with lysozyme the tumor necrotizing activity slightly increased, and after prolonged incubation for one or two weeks the fever producing activity substantially decreased. The interaction with lysozyme was accompanied with changes in the sedimentation: lower molecular weight components appeared. Some fractionation studies were carried out in the moving platform analytical cell in the ultracentrifuge, in collaboration with Dr. William Carroll, Laboratory of Physical Biochemistry, NIAMD.

III. Virus Inhibition

(a) Inactivation of viability of T bacteriophages

T2 bacteriophage virus attacks the receptor cell E. coli by attaching through its tail protein to the bacterial cell surface. This tail protein causes the serological specificity of the virus and it can be inactivated by interaction with antibody. It is a basic protein, similar to lysozyme (Anfinsen and Dryer). It attaches to the negative cell surface, and it leads through an enzymatic process to the penetration of the cell wall by the tail core, and to the eventual injection

of DNA. Our approach, with B. G. Young, was to incubate the bacteriophage with negatively-charged synthetic polysaccharide derivatives, to see to what extent the polyanion would interact with lysozyme and inactivate the viability of the virus by blocking attachment and/or the injection process (ref. 3).

We have found that polyglucose sulfate at a concentration of 2 μg/ml. effectively inactivated the bacteriophage at pH 4.6, while at the same pH sulfuric acid did not affect viability. At somewhat lower pH (4.0) sulfuric acid also inhibited the bacteriophage to some extent, but this inhibition was reversible upon further incubation at neutral pH. The polyglucose sulfate inactivation was not reversible by further incubation at neutral pH, or by increasing the salt concentration, or by adding positively charged macromolecules. Thus, this inhibition is not due to a purely electrostatic interaction with the positive lysozyme. Similar inactivations were achieved with polyglucose carboxyl, but at higher dose levels. The T1 bacteriophage system was even more sensitive to such polyanionic inactivation, and essentially similar inactivation was demonstrated also with T4 and T7 bacteriophages; showing that this type of inactivation of the attachment, or injection, or multiplication of bacteriophage is a general phenomenon.

In studying the mechanism of the bacteriophage T2 inactivation, we found that we do not cause inactivation because of an ion exchange effect of the polyanions. Calcium ion is known to be necessary for the contraction of a protein muscle in the tail of the phage during the injection of the DNA. The polyanionic inhibitor might have been acting through taking up Ca++. But at high Ca++ concentration, more than ten times over the capacity of the polyanion, we could still observe inactivation.

Polyglucose sulfate, labeled by S^{35}, interacted with the T2 bacteriophage directly, while it did not penetrate into the E. coli cell. This suggested that the mechanism is not through blocking some of the processes of multiplication in the cell, but by preventing normal attachment or DNA injection.

When the inactivation was only partial, the residual viable phage had a normal attachment rate to the E. coli. The attachment of the total phage components also was not reduced significantly. These measurements were done with phage where the DNA was labeled with P^{32} and the protein with S^{35}. Experiments of shearing off the labeled attached phage from the E. coli surface by swirling it in a Waring blendor, showed that it was easier to shear off DNA from the inactivated phage than from an untreated virus. This might be because less DNA was injected from the

inactivated system or, because the bacteriophage DNA was more strongly negative in the inhibited phage and it is repelled more by the negative cell surface, and thus it is more easily sheared off. We found no incorporation of labeled P^{32} into the progenies of the bacteriophages.

Our interpretation of the above results is that lysozyme is blocked by the anionic polysaccharide but not sufficiently to prevent attachment, only the penetration and the injection of the DNA. However, a second inactivation mechanism might go on parallelly .The negative DNA in the head of the virus is known to be partially neutralized by positive oligopeptides such as putrescine (Ames). The DNA may change its shape as a polyelectrolyte if the positive putrescine is removed by the negative polysaccharide, making eventual passage through the narrow tail core impossible. That this is likely to be part of the inactivation mechanism was shown in recent experiments with Dr. Rizvi, who demonstrated removal of putrescine without release of DNA from the phage head.

In our inactivation studies with B. G. Young, we observed that a small percentage of the bacteriophage population which remained viable, gave progenies which were more resistant to further inactivation. This indicated that the inactivation might be a useful tool for genetic studies.

B. G. Young also observed that when the bacteriophage was completely inactivated with a sufficient amount of antibody, low concentrations of polyglucose sulfuric acid caused actually a reactivation of a certain percentage of the bacteriophage.This can be accounted for only by assuming direct interaction between the antibody and polyglucose sulfate. This indicates interesting possibilities in antibody fractionation, and in studying antigen antibody interactions by using charged polyelectrolytes.

In addition to the above discussed polyanionic inactivation of T2 bacteriophage, B. G. Young observed (Ref. 4) an irreversible inactivation of the virus at elevated pH (8.5-10.0) in the presence of dialdehyde derivatives obtained from mono- or polysaccharides after periodate oxidation. It was necessary to have at least two aldehyde groups in the same molecule to have this inactivation, but high molecular weight was not a necessary requirement. Mechanism studies with labeled bacteriophage showed that the attachment of protein S^{35} and DNA P^{32} was reduced. The reduction in viability was independent of the virus concentration. Inactivation increased greatly at elevated temperatures.

In the normal receptor sites of the E. coli the cell wall contains many carbohydrate aldehyde groups connected with each other in the same polysaccharide structure. We postulated that the synthetic dialdehydes compete with the reaction between these groups and the amino groups in the basic attachment protein (lysozyme) by forming probably a double Schiff base link, forming a bridge between the neighboring protein chains. Now the DNA cannot pass through the crosslinked protein layer. At the same time the attachment must be also reduced because the positive charge of the free amino groups is also reduced during the formation of the Schiff base.

The bacteriophages were used as models to study quantitatively the changes which polyelectrolytes may bring about in the surface proteins of the virus. The virus protein is obviously important as the instrument responsible for the initiation of infection. Thus far we have found interesting and effective ways to block the infective process in the bacteriophage system. We intend to extend our findings to mammalian viruses especially to tumor viruses.

(b) Tumor viruses

We chose the (Stewart-Eddy) polyoma virus for further studies, to learn how the protein coat participates in the infection by this virus. Of course, first we are in need of a quantitative and rapid assay. We chose the method of Dulbecco and Sachs to produce plaques in a monolayer of embryonic mouse tissue culture. B. G. Young is developing this technique to make it a suitable assay for our need.

III. Program on Synthetic Polysaccharides

To study interaction and inactivation of negatively-charged biological macromolecules, we decided to prepare water soluble positively-charged (basic) derivatives of synthetic polysaccharides. J. W. Wood screened the various methods previously found useful for introducing amino groups into sugars and polysaccharides. In the course of this work he developed new methods, which lead to the introduction of unprecedentedly high amounts of amino groups into polysaccharides. His most promising leads were: (1) the reduction of tri-β-cyanoethyl derivative of polyglucose with lithium aluminium hydride in tetrahydrofurane; (2) the reaction of 1-N-diethylamino-2,3-epoxypropane with polyglucose in aqueous sodium carbonate solution. With both of these methods, he obtained basic derivatives with graded substitution by altering the reaction

conditions and the molar ratio of the reagents employed.

In extending our work on the polycondensation of sugars, P. Maury is engaged in an attempt to polymerize thymidilic acid to form a long chain linear 3'-5' linked phosphate double ester of the nucleotide. She approached the problem by first blocking (in the form of the trityl derivative) the thymidine at the number 5' hydroxyl of the deoxyribose unit, and then phosphorylating the only remaining hydroxyl at the number 3' position, using dibenzylchlorophosphonate (Todd). She plans to take off the trityl groups by hydrogenation in the next step, and obtain 3'-thymidilic acid (the phosphate at 3' position), with the primary 5'-hydroxyl now open. This 5th hydroxyl is more reactive than the secondary hydroxyl at the 3' position, which is free in the 5'-thymidilic acid, the normal enzymatic breakdown product of DNA. Consequently, there is a better chance of polycondensation of 3'-thymidilic acid in the presence of water absorbing and proton donating reagents to high molecular weight 3-5' linked polyesters. Such 3'-5' linked long chains of polynucleotides from a single type of nucleotide would be synthetic models for a simplified DNA.

The previously prepared synthetic polysaccharide derivatives, especially the acid derivatives, were in great demand: several dozen research investigators from various institutions requested samples, some of them following through on our leads on enzyme inhibition.

Some of the synthetic polysaccharides (synthetic polyrhamnose and polymaltose) were shown to possess considerable lipemia-clearing activity without demonstrable toxic or anti-coagulant activity, and some others inhibited mitosis in tissue culture. (Results of Professors Meier and Di Marco). The general aim is to learn more about the nature of the structural and macromolecular properties necessary for the different biologic effects of polysaccharides. In a cooperative undertaking with Prof. Meier's group we plan to incorporate lipoid substances beside the negatively-charged groups into the synthetic polysaccharides, to have a compound more similar in structure and possibly in biologic activity to the bacterial endotoxins. Other work with Di Marco is aimed to find selective inhibitors of growth processes, by using some of our negatively charged polysaccharide derivatives.

Lastly, these synthetic charged polysaccharide derivatives can be usefully employed in fractionation to obtain at a certain pH a complex precipitate with a biological macromolecule. After

centrifugation and separation from the other soluble components, the precipitate can be easily dissociated again by changing the pH or the salt concentration. Such leads were developed for example on the fractionation of the endotoxin detoxifying component of Landy, and are going to be followed through by Dr. Oroszlan.

Course of project

Some indications on further course of research are given in the preceding section. The enzyme, coenzyme and substrate interaction studies, the enzyme inhibition and especially the enhancement studies will be continued. The fundamental studies will be designed to obtain more information on the nature of the interacting and orienting forces between enzymes and substrates.

In the virus studies, the bacteriophage inactivation mechanism will be further studied, with special reference to the possible changes in the DNA function due to the polyelectrolyte effect. We will investigate whether the presence of basic oligopeptide is necessary to the function of the DNA as a template during the reproduction process inside the cell. We also plan to study the nature of the resistance of certain percentage of the bacterial viruses to the polyelectrolyte inactivation. Most of the effort, however, will be concentrated to initiate a study on the role of the proteins in the infective process of the polyoma virus, with the hope of finding means of preventing the spread of the tumor virus from cell to cell.

The synthetic program will be directed to obtain new drugs--polysaccharide complexes, where the drug useful in cancer chemotherapy will be shielded by the polysaccharide. This polysaccharide coat will be designed so that the drug would be expected to be incorporated into the cancer cells at higher concentration than into the normal cells. The work on the polycondensation of 3-thymidilic acid will be continued. Interaction between polynucleotides and basic polypeptides will be also explored.

The fractionation and identification of the mammalian tissue resistance factors will be approached first by concentrating on the isolation of the endotoxin detoxifying component of tissues. This work might be extended into a study of the cytotoxic components in the tissues possibly directed against tumor cells. Such work will be carried out in collaboration with the Polysaccharide Section of this Laboratory.

Significance to Biomedical Research and to the Program of the Institute

The importance of understanding better the common features of enzyme reactions and to learn more on the forces which initiate the oriented interaction between enzymes and their substrate was mentioned above. In vitro studies on the effective inhibition and stimulation of some enzyme systems may lead to better understanding of the complex and interrelated reactions in vivo. Our polyelectrolyte inhibition is believed to be a simplified model of how some of the enzyme regulation might occur in vivo. Of course, the presence of numerous regulatory functions, macromolecules etc. in vivo may make our approach appear rather naive. However the observations on enzyme stimulation of "impure" systems brought to our attention one possible way in which the control of enzyme rates may be achieved in vivo through many interrelated equilibriums between charged biological macromolecules. There are known differences in enzyme rates between neoplastic and normal tissues. The information from our studies may point out a general cause of upset of controls, and may point a way to the re-establishment of normal enzyme rates in neoplastic tissues.

The bacteriophage inhibition studies were carried out as a prelude to the work on tumor causing viruses. Such quantitative model studies in the bacteriophage system showed us several possible ways of interfering with the role of the protein, which is the infection initiating part of the virus. We believe that better understanding of the role of the bacteriophage protein in the infective process, and of the antibody neutralization of the phage, already gave us leads, and will continue to give us more leads, on how the infectivity and the spread of the tumor viruses from cell to cell might be controlled through similar processes.

Serial No. NCI-262
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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project.

(1) Mora, P. T., Wood, J. W. and McFarland, V. W.: Synthetic Polysaccharides. V. Polymerization of Various Aldoses. J. Am. Chem. Soc. 82: 3418 (1960).

(2) Mora, P. T., Young, B. G. and Shear, M. J.: Effect of Polyanion Charge Density on the Inhibition of Ribonuclease. Macromoleculare Chemie 38, 212 (1960).

(3) Young, B. G. and Mora, P. T.: Viability of T2 Bacteriophage After Interaction with Negatively Charged Macromolecules. Virology 12, 493 (1960).

(4) Young, B. G. and Mora, P. T.: Reduction of Viability of T2 Bacteriophage with Polyaldehydes. Biochem. Biophys. Acta. In press.

Honors and Awards relating to this project.

There was a special symposium on Synthetic Polysaccharides at the 138th National Meeting of the American Chemical Society in New York City in September 1960. P. T. Mora was invited to organize this symposium and to preside. He also lectured by invitation at the Universities of Birmingham, Ala., Edinburgh, Scotland (International Symposium on Polysaccharides), Milan, Italy, and here at Washington, D. C. at Georgetown University.

Serial No. NCI-261
1. Lab. Chemical Pharmacology
2. Polysaccharide Section
3. Bethesda 14, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: The Role of Polysaccharides and Other Naturally Occurring Substances in Immunologic Phenomena, both Basic and Related to Neoplastic Processes.

Principal Investigator: Dr. M. Landy.

Other Investigators: Drs. W. R. Keene (now at USPHS Hospital, Boston, Mass.), J. G. Michael, A. L. Notkins, M. J. Shear, R-J. Trapani and J. L. Whitby (Guest Scientist, Univ. of Birmingham Medical School, Birmingham, England).

Cooperating Units:

(a) Laboratory of Biochemistry, NCI: Dr. M. W. Woods, Serial No. NCI-334.

(b) Laboratory of Biology, NCI: Dr. M. Potter, Serial No. NCI-427(a).

(c) General Medicine Branch, NCI: Drs. E. Frei and R. Mechanic, Serial No. NCI-5737(c).

(d) Rocky Mountain Laboratory, NIAID: Dr. E. Ribi.

(e) Biochemistry Branch, Armed Forces Institute of Pathology: Dr. V. S. Waravdekar.

(f) University of Florida School of Medicine: Dr. E. Suter.

(g) State Bacteriological Institute, Stockholm, Sweden: Dr. B. Björklund.

Man Years (Calendar Year 1960)		Patient Days
Total:	7-3/4	None
Professional:	4-1/4	
Other:	3-1/2	

Project Description:

Objectives:

Investigation of humoral and cellular factors in the host involved in natural immunity to infection and experimental neoplastic disease. Study of the mechanisms by which these factors are influenced by polysaccharides and by other substances of natural origin. Comparison of the antigenic properties of cancer and of normal cells.

Methods Employed:

Biochemical, immunological, microbiological, and physiological, modified as required to meet the specific needs of particular problems.

Major Findings:

1. Human Carcinoma Antigen (with Trapani and Björklund):

It was reported by Björklund that human carcinomas have antigenic properties different from those of normal human tissues. By prolonged immunization of horses with preparations of pooled human carcinomas, an antiserum cytotoxic for HeLa cultures was obtained by him. This cytotoxicity, which was not affected by absorption with normal human tissues, was removed by absorption with human carcinoma tissue or by HeLa cells. We have initiated an investigation, in which we sought to confirm the principal findings of Björklund and to explore the possibilities of developing serological procedures for more extensive work with such antigenic preparations. Our serological experiments have indicated that it is possible, under appropriate conditions, to "coat" tannic acid treated erythrocytes with preparations extracted from HeLa cells with buffer at pH 8. The treated erythrocytes were agglutinated to high titer by horse antiserum against pooled human carcinomas. It was suspected that the antiserum also contained antibodies against normal tissue antigens. The presence of such antibodies was demonstrated with the use of tanned erythrocytes coated with pooled normal human serum. These cells were also agglutinated to high titer by the horse antiserum. We found that practically all of the antibodies reactive with human serum coated erythrocytes could be removed from the antiserum by absorption with cell suspensions of normal human tissues. However, the reactivity of such absorbed serum with HeLa antigen-coated erythrocytes, and its cytotoxicity for HeLa cultures, were not

discernibly affected. On the other hand, analogous absorption of the antiserum, with Björklund's antigen preparations derived from human carcinomas, removed cytotoxicity for HeLa cultures and reactivity for erythrocytes coated with HeLa antigen; it is noteworthy that reaction with erythrocytes coated with normal human tissue antigens remained unchanged. The absorption technique was controlled by parallel treatment of an antiserum prepared in rabbits by immunization with normal human serum. This antihuman serum was cytotoxic for HeLa cells and agglutinated erythrocytes coated with HeLa antigen or with preparations from normal human tissues. However, these properties were no longer evident after the serum had been absorbed with normal human tissues.

2. Antibody Production by Mice with Plasma Cell Tumors (with Potter):

Exploratory experiments were initiated on the sera of mice in which plasma cell neoplasms had been induced with paraffin oil and bacteria. We sought to determine whether the abnormally high levels of beta and gamma globulins in the sera of these animals represented antibody or disoriented protein. The serum of mice normally contains, in low titer, a broad spectrum of individually specific antibodies to Gram-negative bacterial species. As far as can be determined, these antibodies are the result of antigenic stimuli supplied by continuous contact of those animals with Gram-negative bacteria. It was reasoned that in the presence of this persistent, multiple, natural antigenic stimulus, the greatly increased synthesis of serum globulins, usually associated with antibodies, might be reflected in markedly increased levels of antibody to these bacterial species. Employing the sensitive and objective bactericidal assay for antibodies to Salmonella, it was found that sera of mice with these plasma cell neoplasms contained no greater amount of Salmonella antibody than that present in normal mice. Since the increased level of globulins did not reflect enhanced antibody response to bacteria, the reason for these globulins is still obscure.

3. Lethality of Normal Serum for Mouse Tumor Cells (with Trapani, Michael, Achinstein and Woods):

Systematic investigation of the antibody-complement system in normal serum lethal to mouse tumor cells, reported on last year, has been largely completed. The general findings were that normal sera from many animal species exhibited in vitro a lethal effect on ascites cells of Sarcoma 37 and a considerable

number of other tumor cells of mice. This in vitro activity of serum on the tumor cells of a heterologous species was manifested by: failure to proliferate in a susceptible host; altered permeability to eosin; morphologic changes; and cessation of glycolytic activity. The system in serum responsible for these effects consisted of a natural antibody and complement. This antibody had specificity for an antigen present in tumors and lymphoid tissues of mice. In continuation of the study the failure of fresh human serum to exert a comparable effect on tumor cells in vivo was shown to be due to uptake of the antibody by normal lymphoid cells of the mouse. The most convincing of a series of experiments was one in which serum injected into mice intraperitoneally, and aspirated 5 minutes later, was found to retain fully its complement activity but to be devoid of the antibody. In this regard it was indistinguishable from serum absorbed in vitro with Sarcoma 37 or mouse spleen cells. The absence of activity in the serum of 7 of the 17 species tested was puzzling at first. The simplest explanation was that the required antibody was absent. However, in absorption experiments it was demonstrated that S-37 cells exposed at 4° C. to an inactive serum (horse or guinea pig, for example) were then susceptible to the lethal effect of complement, as provided by human serum from which the antibody had previously been absorbed with S-37 cells. It was thus evident that the inactivity of serum from a number of species was not due to lack of antibody. On the other hand, S-37 cells which had taken up antibody from human serum were not killed by subsequent exposure to the complement in the serum of guinea pig or horse.

That this lack of cytotoxic activity in the sera of certain species was not limited to the tumor cells under test was shown in experiments in which the lytic properties of these inactive sera against mouse erythrocytes was determined. It was found that, as with the tumor cells, sera of species such as guinea pig, sheep, horse and hamster did not lyse mouse red cells. These sera did, however, contain hemolysin for mouse erythrocytes since an appropriate source of complement (human serum absorbed with mouse red cells) brought about lysis. On the other hand the same sera did lyse red cells of species other than mouse, viz., rabbit, human, etc., showing that these sera do contain a functional antibody-complement hemolytic system. Moreover, when mouse red cells were coated with endotoxin, from any of a number of Gram-negative bacterial species, and then exposed to serum from guinea pig, for example, the cells lysed. This showed that the class of "inactive" species did in fact possess a functional antibody and complement

against a bacterial component. Finally it was shown that sera from two "inactive" species such as hamster and guinea pig, when combined in equal proportions would cause lysis of mouse red cells.

The findings have made it evident that serum from various species may differ strikingly in the properties of the components of complement which they contain.

4. Host Reaction to Carcinogen (with Keene and Notkins):

Study of the effect of a carcinogen on the natural immunity of the mouse was continued. CAF_1 mice were given a single i.v. injection of methylcholanthrene (MCA) in Nujol; controls received the vehicle alone. The dose levels employed were 0.1 and 0.01 mg/kg. Effects on body weight, total leucocyte count, hematocrit, and RES activity (as determined by clearance of colloidal carbon from the circulation) were determined at weekly intervals for 6 weeks following MCA. Small numbers of mice were sacrificed at these intervals in order to assess any gross changes in tissues. No significant alteration in these parameters was noted. However, by the third week after treatment a marked decrease in size and weight of the spleen was evident in the mice that had received 0.1 mg MCA/gm. Several weeks later the size and appearance of the spleens had returned to normal. Six weeks after MCA treatment skin grafts from C-57 Black mice were applied to MCA treated and control mice in order to determine whether transplantation immunity had been affected by the carcinogen. No difference was discernible in the rate at which the grafts were rejected by the MCA and the control groups.

5. Relationship of Chemical Composition to Biological Activities of Endotoxins (with Ribi):

Interest in the extent to which firmly bound lipid (lipid A) accounts for the characteristic host-reactive properties of endotoxins has continued unabated in many institutions here and abroad. Our investigation of the relationship of chemical composition to biologic activities of endotoxin has been continued. The concept has been developed by other workers that, were it possible to disperse the free lipid A in water in a manner comparable to the original state as it was bound to the polysaccharide carrier, this free lipid would then display biological activity similar to the endotoxin complex. To assess the validity of this thesis, which has attracted widespread interest and considerable

acceptance, the biological potencies of a number of lipid A preparations were determined in parallel with those of their parent endotoxins, employing quantitative bioassays based on the following host responses: fever, increased resistance to infection, tumor damage, primary inflammation of skin, and lethality. Without exception, preparations of lipid A displayed only a small fraction of one percent of the biological activity of the endotoxins from which they were derived. We then dissociated lipoidal fractions from endotoxin by non-hydrolytic procedures without appreciable reduction in the potency of the endotoxins. These lipids exerted biological effects of the same low order as lipid A isolated by acid hydrolysis, a method which led to destruction of the potency of the endotoxin. Data were obtained which indicated that lipid A was of a heterogeneous nature.

Endotoxins of low lipid content, previously prepared from S. enteritidis by the aqueous ether procedure, we treated further for removal of bound lipid with non-hydrolytic methods such as boiling with monochlorobenzene-ethanol. Such endotoxins, containing less than 3% lipid A, were as potent as those prepared by the well-known phenol-water or Boivin procedures which yield products containing 20-35% lipid A. By refinement of methodology, endotoxins were prepared which contained no more than 0.5% nitrogen and 1.7% fatty acid ester. The analytical values for these potent endotoxins did not differ appreciably from those of the well-characterized haptenic polysaccharide as regards nitrogen, phosphorus, aminohexose, and fatty acid ester.

It has been assumed by some workers that firmly bound lipid liberated by acid hydrolysis is of lower biological activity than the original endotoxin because the lipid is separated from the hydrophilic polysaccharide carrier. Accordingly, a study was made of the rate at which biologic activities diminished in relation to the release of firmly bound lipid during progressive hydrolysis of S. enteritidis endotoxin with 0.1 N acetic acid at 100°C. Each of five quantitative bioassays for endotoxin revealed that biological activity had been reduced to negligible proportions prior to any significant liberation from the endotoxin of firmly bound lipid. Consequently the loss of biologic potency could not be explained by the separation of water insoluble lipid from the endotoxin complex.

6. Effects of Endotoxin on Metabolism of Mammalian Cells (with Woods):

Studies on the metabolic effects of bacterial endotoxin on mammalian cells were continued. We showed that, under proper conditions of measurement, the sensitivity of aerobic and anaerobic glycolysis in vitro of various mammalian cells to inhibition of steroids indicated the extent to which the particular cells were subject to growth restraint by the host via "anti-insulin" hormones. These act on the hexokinase reaction to inhibit glycolysis and growth. The material studied thus far includes various mouse tumor cells, human and rabbit marrow and neutrophilic granulocytes, mouse peritoneal macrophages, spleen and kidney cells.

Bacterial endotoxins and/or insulin in low concentrations act on glucose metabolism in such a manner that both aerobic and anaerobic glycolysis are stimulated. The lowest effective concentration of endotoxin is approximately 0.0001 µg/ml.; 0.3 µg/ml. generally provides maximal effect. Between these extremes the degree of stimulation is fairly well related to the concentration of these polysaccharide complexes. While endotoxins and insulin undoubtedly have different overall effects on the cells, these studies have shown that there are certain important similarities in the biological specificity of their glycolytic action.

Under in vitro conditions a wide range of steroids could be used to assess the sensitivity of glycolysis in particular to hormonal, viz., insulin-anti-insulin regulation. The sex steroids testosterone, estradiol, diethylstilbestrol and progesterone were the most efficient. Cortisone dihydrocortisone and prednisolone were somewhat less active.

A considerable body of data has been accumulated which shows that the capacities of a series of endotoxins (from different Gram-negative genera, isolated by different procedures, and having different potencies in eliciting characteristic effects in the host) correlated well with this stimulation of glycolysis in vitro. Moreover, experiments on the degradation of endotoxin by humoral and intracellular enzymes of the host, or by progressive acid hydrolysis, resulted in a loss of capacity to stimulate glycolysis. In this regard the changes paralleled elimination of activity to elicit host responses.

These correlations have been extended to in vivo situations where it was found that the administration of endotoxin to normal mice brought about significant changes in the metabolic activity of host cells. Within hours of the injection of a single small dose of endotoxin, peritoneal macrophages displayed a marked increase in glycolytic activity in vitro. As regards the quantity of endotoxin required to elicit this effect and the magnitude of the stimulation, the in vivo effect of endotoxin was more impressive than the in vitro. The reasons for these correlations and the locus of action in the cell remain to be determined.

7. Bioassay of Endotoxin (with Keene):

A study was made of the pyrogenic response of the rabbit for application to the bioassay of endotoxin. The most reliable dose related measure of the febrile response was the "fever index", inasmuch as it represents an objective and reproducible expression of both height and duration of the fever curve. The febrile response of untreated rabbits to endotoxin, as measured by the fever index, varied appreciably even when a number of precautions were taken to minimize variation. Nevertheless, when the febrile response of groups of four or more animals were averaged, the mean responses were found to be constant and the dispersion of values was then well within acceptable limits. The minimal pyrogenic dose has frequently been used to compare the pyrogenic potencies of different endotoxin preparations. However, it was shown that more precise comparisons are obtained when an amount of endotoxin is used which is sufficient to produce febrile responses that fall on the linear portion of the dose response curve. For the assay based on fever index, this was the minimal dose necessary to produce a fever index of 40 cm^2.

8. Enzymatic Degradation of Endotoxin (with Keene):

The inactivation of endotoxin by blood plasma described in previous annual reports was found to have the characteristics of an enzyme-catalyzed reaction. It was shown that reduction of pyrogenic potency for rabbits was dependent upon pH, adequate concentration of plasma and endotoxin, as well as upon the time and temperature at which endotoxin and plasma were interacted in vitro. In addition, minute quantities of a number of divalent cations, especially calcium, non-specifically inhibited the degradation of endotoxin by plasma. The identity of the reaction products remains to be defined. In this work, the progress

of the enzyme reaction was followed by measuring residual substrate (endotoxin) concentration in the pyrogen assay. Employment of this assay as a measure of residual endotoxin was based on the inference that the residual fever producing substance in plasma-endotoxin reaction mixture was unaltered endotoxin. In the previous annual report it was suggested that the residual pyrogen of incubated plasma-endotoxin reaction mixture had the pyrogenic properties of endogenous serum pyrogen. It has since been demonstrated that animals tolerant to endotoxin are also tolerant to the residual pyrogen. In addition, daily injections of the residual pyrogen induced endotoxin tolerance. Since the most distinctive quality of endogenous pyrogen is that it is equally pyrogenic for normal and endotoxin-tolerant animals it would appear that the residual pyrogen of incubated plasma endotoxin reaction mixtures is not endogenous pyrogen.

9. Intracellular Endotoxin Degrading Enzyme (with Trapani and Waravdekar):

Studies on the endotoxin detoxifying agent in animal tissues were continued. The preliminary findings reported last year, that such activity was present within cells of certain rabbit organs, were extended along several lines. With the recognition that the endotoxin assay utilizing the febrile response of the rabbit was quantitatively superior to the tumor damage assay used in this study last year, most of the experimental work was repeated with the use of the pyrogen assay. It was shown that inactivation in vitro of endotoxins by cell-free homogenates of perfused rabbit liver displays the characteristics of an enzyme-catalyzed reaction. This intracellular enzyme was relatively heat labile and was inactivated at 50°C. within 20 minutes. It was effective over a wide pH range but, under limiting conditions of enzyme concentration and time, it had a distinct pH optimum of 9-9.5. Its action was temperature dependent, diminishing rapidly at temperatures below 37°C. With appropriate concentrations of enzyme and substrate, considerable inactivation of endotoxin was evident within 5 minutes. Unlike the enzyme present in blood, this intracellular enzyme was not demonstrably influenced by the presence of calcium ions. The enzyme preparations derived from rabbit liver displayed good endotoxin-degrading activity, but the search for analogous activity in other tissues of the rabbit has been, for the most part, unsuccessful. Thus, brain, connective tissue, spleen, myocardium and adrenals were consistently inactive, while enzymatic activity was at times discernible in lymph nodes, spleen and kidney, but this has varied from one preparation to another.

Intracellular enzymatic activity, with similar properties, was derived with the use of another procedure. It has been reported that when whole cells are incubated under appropriate conditions an efflux of intracellular enzymes into the surrounding medium takes place. The conditions of incubation were those that influenced cell metabolism in such a way that cell permeability was altered. This principle was applied to rabbit liver cell suspensions prepared with the use of tissue press and Snell cytosieve. The cells were washed with Ringers solution and extracted with buffer (high in potassium) for 1 hour at 37°C. Extracts obtained in this manner were potent in destroying pyrogenicity and tumor-damaging activity of bacterial endotoxin. In contrast to liver homogenates these preparations were low in total solids and nitrogen, and were water clear.

10. Inactivation of Endotoxin by Leucocytes (with Notkins):

Since polymorphonuclear leucocytes are important in host defense the possibility that these cells detoxify endotoxin was explored. Rabbit granulocytes, obtained by peritoneal infusion of saline, were employed. A homogenate of 1 x 10^8 leucocytes incubated for 1 hour at 37°C. with 100 µg of Serratia marcescens endotoxin caused a reduction of more than 50% in the pyrogenicity of this endotoxin. The leucopenic action of endotoxin was also annulled as was its tumor-damaging activity. The inactivation by leucocyte homogenates was temperature and time dependent, but a pH optimum was not demonstrated. The detoxifying agent from granulocytes was destroyed by heating for 2 minutes at 100°C., or for 1 hour at 70°C., but withstood 1 hour at 56°C. When fractionated by centrifugation, the supernatant was inactive, the sediment showed some activity, but the entire homogenate was the most effective. Other formed elements of the blood, including human erythrocytes, platelets and rabbit monocytes did not inactivate endotoxin.

11. Release of Enzyme by Host Cells exposed to Endotoxin (with Notkins):

It has been reported that endotoxin acts by damaging granulocytes, thereby leading to release of endogenous pyrogen from these cells. If this is so, release of other intracellular materials should also occur. Accordingly, evidence was sought for the release of lactic dehydrogenase (LDH), an intracellular enzyme which can be measured accurately, following administration of large amounts of endotoxin to mice. No elevation in serum LDH

occurred after injection of endotoxin even in amounts approaching lethal levels. Nor was there any significant release of LDH from mouse peritoneal macrophages when these cells were incubated *in vitro* with endotoxin. Thus, as measured by release of LDH, no evidence was obtained in support of the thesis that endotoxin, in pharmacologically effective dosage, produces damage to these cells.

12. Leucocytic Response of Humans to Endotoxin (in collaboration with Mechanic and Frei):

The granulocytic leucocytosis produced by the injection of bacterial endotoxin is believed to reflect the state of the bone marrow granulocytic reserve. The possibility was considered that this reaction might prove a more convenient and reliable method of assessing the functional state of the bone marrow than the peripheral leucocyte count and examination of aspirated marrow. This test might therefore become a useful adjunct to chemotherapy programs involving the administration of drugs which depress marrow activity. Administration of Pyrexal (purified *Salm. abortus-equi* endotoxin) to normal subjects at a level of 0.003 µg/kg. elicited granulocytopemia and febrile responses, followed by a leucocytosis. With progressive reduction in the dose of endotoxin, the dose for threshold febrile response was found to be approximately 0.007-9 µg/kg. This level produced leucocytosis without an initial diminution in granulocytes. Indeed, 0.0002 µg/kg., an amount insufficient to evoke a significant rise in temperature, still elicited leucocytosis. In three subjects, tolerance to fever was induced by the daily injection for 4 or more days of 0.005 µg/kg. However, the capacity to respond with leucocytosis continued unabated.

This line of work is reaching the point where it may be worthy of exploration in clinical situations.

13. Alteration of Bacteria coated with Endotoxin (with Michael):

Last year we reported that antigenically related strains of Gram-negative bacteria, differing in their susceptibility to the lethal action of normal serum, varied in their content of endotoxin, i.e., serum resistant strains contained more endotoxin than did their serum-sensitive counterparts. To obtain further evidence for the role of endotoxins in this regard, Gram-negative bacteria were exposed to endotoxin *in vitro* and their

susceptibility to serum was examined. The coating of bacteria with endotoxin which resulted was dependent on the length of exposure, relative concentrations of bacteria and endotoxin, the interaction temperature, and the physiological state of the bacterial cells. Bacteria in the log phase of growth took up endotoxin from the medium much more effectively than cells in the stationary phase. The lethal action of serum on the bacteria was, to a considerable extent, augmented or diminished depending on the quantity of endotoxin taken up by the cells. Small amounts of endotoxin resulted in increased susceptibility, whereas large amounts caused high resistance to serum.

It was found that endotoxin could not be utilized as the sole source of energy (carbon) for growth by Gram-negative species. However, after hydrolysis with HCl, the split products of the endotoxin were readily utilized, the bacteria grew well and the antigenic specificity of the haptenic polysaccharide present in the hydrolysate was incorporated in the bacterial cells.

14. Modification of Host Response to Endotoxin (with Trapani and Suter):

For a better understanding of the nature of the effects of endotoxin on mammals, it was considered desirable to explore the various means by which host susceptibility to endotoxin is modified. Prior treatment with endotoxin which induces a state of refractoriness or tolerance is the subject of intensive study. However, the kinds of treatment which greatly increase susceptibility are less well defined. These are: RES-blockade, adrenalectomy, tumor implantation, and administration of BCG vaccine. As a preliminary to more extensive work seeking to determine whether these diverse treatments produce in the host any change in common, it was sought to determine whether combination of these treatments would be either additive or synergistic. Accordingly, CAF_1 mice were injected i.v. with 50×10^6 BCG, Sarcoma 37 was implanted 4 days later, and on day 10 they were challenged with S. enteritidis endotoxin. The BCG treated mice were highly susceptible to the lethal action of endotoxin, as were the Sarcoma 37 implanted mice; the LD_{50}s were 0.5 µg and >3 µg, respectively. However, the LD_{50} for mice that had been subjected to both effects was significantly higher than that of animals with BCG alone. There was no synergism; the effects were not additive, and the findings suggested that the tumor interfered with the action of BCG.

15. Quantitative Assay for Natural Bacterial Antibody (with Whitby and Michael):

In the course of investigating alterations in the somatic endotoxic complex of Gram-negative bacteria which, as we reported last year, occurs during the serum bactericidal process, it was observed that very small amounts of antibody sufficed for bactericidal activity. These findings suggested that the bactericidal reaction could be utilized to develop a sensitive assay for antibody to various Gram-negative bacterial species. Among the many natural antibodies invariably present in normal serum of mammals those reactive with Gram-negative bacteria are considered important in normal resistance to microbial infection. There have been conflicting reports on the origin and specificity of these antibodies, principally because of the lack of discriminating techniques for measuring them in their low concentration in normal serum. In our tests for these normal antibodies, pooled normal human serum, absorbed at 4°C. with the appropriate heat-killed Gram-negative bacteria, served as the complement source. The absorbed serum was non-bactericidal against the homologous bacterium. Addition of normal mouse serum, itself non-bactericidal, restored bactericidal activity for enteric species such as Salm. typhosa and E. coli. Under appropriate conditions of test the magnitude of killing was a function of serum concentration (antibody). To obtain consistent, dose-related bactericidal effect, the critical factors were size of the bactericidal inoculum (1×10^6), time and temperature of incubation and relative proportions of complement, antibody and bacteria in the reaction mixture. That this test for antibody was specific was shown by absorptions with homologous and heterologous serotypes, and by experiments in which the bactericidal effect of the system was blocked by the addition of purified homologous somatic antigen, but not by heterologous endotoxin. Tests with an immunochemically calibrated antiserum against Salm. typhosa showed that the smallest amount of antibody measurable by the method was 0.0002 µg antibody nitrogen, and that 0.001 µg provided marked bactericidal effect. On this basis normal mouse serum was estimated to contain approximately 0.01-0.02 µg antibody N (against S. typhosa) per ml. The method, which is simple, specific and objective thus has the further advantage of being a much more sensitive assay for these bacterial antibodies than any of the methods presently available.

Significance to the Program of the Cancer Institute:

Information on the identity of humoral and cellular agencies of the host participating in natural and induced immunity to infection or neoplastic disease are essential to an understanding of these processes. The utilization of substances which, upon administration to the host, bring about significant alterations in the level of natural or induced immunity, represents a major avenue of approach to this complex problem.

Proposed Course of Project:

1. Studies on antigen preparations derived from human carcinomas will be continued (with Trapani and Björklund). Horse anti-human carcinoma serum, from which antibodies to normal human tissue antigens have been removed by controlled absorption, will be employed as the key reagent in serological studies. Human carcinomas and HeLa preparations will be fractionated in an attempt to obtain more specific antigen preparations capable of coating tanned erythrocytes for reaction with this absorbed serum.

2. It is proposed to investigate (with Potter) the influence of plasma cell tumors on the capacity of mice to produce specific antibody. Special attention will be directed to the response of these animals to antigen injected at the time the mice are treated with adjuvant-Staphylococcus mixture. The antigens to be employed, because of their persistence in host tissues, are acetylaminogalacturonic acid polymer (Vi) and pneumococcal polysaccharides.

3. Employing the bactericidal assay for normal antibody to Gram-negative species, it is planned to investigate (with Whitby and Michael) the origin and specificity of natural antibodies to enteric bacteria. It is anticipated that use of germ-free mice and rats will provide the crucial information. The means by which injection of endotoxin produces in mice a temporary state of increased resistance to infection with Gram-negative pathogens will be reinvestigated from the standpoint of significant changes in the level of these natural antibodies. Evidence for stimulation of macrophages, the other component of the principal host defense system, will also be sought.

4. It is planned to ascertain the nature of the difference in biologically potent, purified endotoxin and haptenic polysaccharide which have the same analytical values (with Ribi).

These products will be compared as regards the following: molecular size and homogenity in the ultracentrifuge; identity of components as revealed by chromatography of their hydrolysates; immunological homogenity as determined in quantitative precipitin and agar gel diffusion tests.

5. Information will be sought on the nature of the degradation products of endotoxin interacted with host enzymes (with Ribi). It is proposed to inactivate highly potent endotoxin with enzyme preparations derived by extraction of washed rabbit liver cells with appropriate buffer. The loss of endotoxic activity will be assayed and the reaction mixtures subjected to chromatography. Control chromatographic analyses will be made of the enzyme preparations and of the acid hydrolyzed endotoxin.

6. Isolation of antibodies will be attempted (with Hirata) by precipitating antibodies with specific adsorbents and dissociating the complex.

Attempts will be made to estimate the total of precipitating and non-precipitating antibody in serum by tagging the antigen with radioactive isotopes and precipitating the soluble antigen-antibody complexes by the use of specific adsorbents made against the globulin, i.e., against the antibody. A quantitative estimation of the number of antibody combining sites in a given volume of serum will be made by radioassay of the precipitate.

The finding that pyrophosphate inhibits precipitation of antigen-antibody complexes will be investigated to ascertain the mechanism of this inhibition.

7. Tissue specific antibodies can be elaborated in animals by the technique of immunological tolerance. It is proposed (with Hirata) to isolate tissue specific antigens in combination with specific adsorbents prepared from specific antibodies. Dissociation of the complex should result in the release of tissue specific antigens.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Landy, M., Trapani, R-J., and Rosen, F. S. Inactivation of Endotoxin by a Humoral Component. VI. Two Separate Systems Required for Viable and Killed Salmonella typhosa. J. Clin. Invest. 39: 352-363, 1960.

(2) Landy, M., Michael, J. G., Trapani, R-J., Achinstein, B., Woods, M. W., and Shear, M. J. An Antibody-Complement System in Normal Serum Lethal to Mouse Tumor Cells. Cancer Research 20: 1279-1291, 1960.

(3) Landy, M. Inactivation of Endotoxin by Humoral Systems. Ann. N. Y. Acad. Sci., 1960. In Press.

(4) Keene, W. R., Silberman, H. R., and Landy, M. Observations on the Pyrogenic Response and its Application to the Bioassay of Endotoxin. J. Clin. Invest. in press 41: (February) 1961.

(5) Keene, W. R., Landy, M., and Shear, M. J. Inactivation of Endotoxin by a Humoral Component. VII. Enzymatic Degradation of Endotoxin by Blood Plasma. J. Clin. Invest. in press 41: (February) 1961.

(6) Michael, J. G., and Landy, M. Endotoxic Properties of Gram-negative Bacteria and their Susceptibility to the Lethal Effect of Normal Serum. J. Inf. Dis. In press. 1961.

Honors and Awards relating to this project:

The Head of the Polysaccharide Section received the following invitations to present work of the Laboratory:

(a) Graduate School Lectures at Louisiana State University and Tulane Medical Schools, February 23, 24.

(b) Visiting Scientists' Lecture Series at Bryn Mawr College, August 4, 5.

(c) Symposium on Mechanisms of Antibody Synthesis, Theobald Smith Society, November 15.

(d) Invited by the Society of American Bacteriologists to organize a Symposium on Bacterial Endotoxins for their National Meeting in Chicago, April 25, 1961.

(e) Invited to present lectures on Natural Immunity to medical students and staff of School of Medicine, University of Puerto Rico, San Juan, December 29-30, 1960.

Serial No. NCI 500
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Histogenesis and Pathology of Induced and Spontaneous Tumors of Laboratory Animals

Principal Investigator: Harold L. Stewart

Other Investigator: Katharine C. Snell

Cooperating Units: Dr. David Johnson, NIAMD

Man Years (calendar year 1960):
Total: 6 1/3
Professional: 2
Other: 4 1/3

Project Description

Objectives: To gain knowledge of the etiologic and pathologic factors in the production of neoplasms in experimental animals and to make this information available to other investigators.

Methods: 1. Adrenal cortical carcinoma 494, a transplantable, hormonally-active tumor that originated in the adrenal cortex of an Osborne-Mendel rat, has been studied during the course of 15 transplant generations in intact, mature hosts. Chemical analysis of the tumor tissue for identification of steroid hormones produced by the tumor is being completed by Dr. David Johnson of NIAMD.

2. A long-term study of the effect of age upon untreated rats of six of the NIH inbred strains is being continued. The purpose of this experiment is to enumerate and classify spontaneous tumors occurring in these animals, to study tissue changes with increasing age, and to furnish controls for present and future experiments. To date, necropsies have been performed on 410 of these rats, histologic sections from all have been examined, and preliminary findings have been tabulated.

3. A study of the spontaneous neoplasms occurring in the Mastomys, a South African rodent, is being continued. To date, necropsies have been performed on 154 of these animals, and most of the tissues have been examined histologically. Since transplantation of tumors to untreated Mastomys has been entirely unsuccessful, several tumors have been transplanted to Mastomys pre-treated with various doses of irradiation. A small number of irradiated but otherwise untreated Mastomys have also been examined.

Part A (continued)

4. Monkeys that received injections of carcinogenic substances in the wall of the glandular stomach from 10 to 12 years ago are being necropsied and examined to ascertain whether these substances may have produced gastric carcinoma.

5. Examination of rats from an experiment conducted by Dr. John B. Calhoun suggested that those rats marked for identification with a black hair dye might have had an increased incidence of mammary tumors. Groups of rats and mice in our laboratory are now being painted three times a week with this dye to ascertain whether they develop more mammary tumors than untreated rats.

6. Collaborative work with Dr. Harold P. Morris: Approximately 4000 histologic sections from rats fed either N-2-fluorenylacetamide or N,N^1-2,7-fluorenylenebisacetamide have been examined. A manuscript has been completed describing the effects of these two compounds upon various organs of the treated rats, and three separate manuscripts have been completed on tumors of the salivary glands, glandular stomach, and 6th cranial nerves induced by the oral administration of N,N^1-2,7-fluorenylenebisacetamide.

Major Findings: 1. The biologic behavior of Adrenal Tumor 494 has remained essentially the same as described for earlier generations, that is, tumor-bearing rats have developed polyuria, polydipsia, and degenerative changes in the distal convoluted tubules of the kidney. They have also shown atrophy of the adrenal glands, sex organs, lymphatic tissue, and pituitary gland. Analysis of the tumor tissue by Dr. David Johnson shows that desoxycorticosterone is the major steroid hormone elaborated by the tumor. Analysis of adrenal glands from normal, untreated, Osborne-Mendel rats reveals that, as in other strains, desoxycorticosterone is absent or present in infinitesimal amounts.

2. Preliminary tabulation of tumors found in untreated rats of six of the NIH inbred strains indicates that the most frequent sites for tumors in the various strains is as follows: AxC, testis and anterior pituitary gland; Fischer, testis; Osborne-Mendel, adrenal cortex; Buffalo, anterior pituitary gland and adrenal cortex; M520, adrenal medulla; Wistar, anterior pituirary gland and mammary gland. Findings in this study were used as teaching material in a lecture of the course on Diseases of Laboratory Animals at the AFIP in September 1960.

3. Tumors found in Mastomys have included a few carcinomas of the glandular stomach as well as papillomas of the forestomach, lung tumors, adenomas of the adrenal cortex, granulosa cell tumors and luteomas of the ovary, and lymphomas. The incidence of hepatomas has been unusually high.

4. No significant findings.

Part A (continued)

5. No significant findings.

6. Collaborative work with Dr. Harold P. Morris: The two principal lesions induced in the organs and tissues of rats fed N,N'-2,7-fluorenylenebisacetamide (2,7-FAA) or N-2-fluorenylacetamide (2-FAA) were atrophy and tumors, many of the latter being cancers. These two lesions were usually associated, but sometimes one or the other occurred independently. Both lesions were influenced by the sex and strain of the rat. In general, 2,7-FAA was the more potent carcinogen; tumors of special interest that developed in some rats fed this compound were sarcoma and mixed tumors of the submaxillary gland, adenocarcinoma of the glandular stomach, and intracranial neurilemmoma of the abducens nerve.

Significance: The knowledge gained from a study of neoplasms in laboratory animals should provide a basis for further work on the mechanism of carcinogenesis in human beings.

Proposed Course: It is expected that a manuscript dealing with identification of steroid hormones elaborated by Adrenal Tumor 494 will be prepared for publication. Final tabulation of pathologic findings in the untreated rats of six NIH strains will probably be made, and it is hoped that a report of these findings will be begun. Further collaborative work with Dr. Morris is anticipated, particularly the examination of histologic sections from rats treated with N,N'-2,7-fluorenylenebisacetamide by the intraperitoneal and subcutaneous routes. A manuscript will be prepared to report the induction of adenomatosis in the lungs of DBA mice fed dibenzanthracene. (This experiment was completed before the current year.) The experimental procedures and examination of histologic material in other items enumerated above will be continued.

Part B included: No

Serial No. NCI 502
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Histogenesis and histopathology of cutaneous neoplasms.

Principal Investigator: John H. Edgcomb

Other Investigator:

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 1
Other:

Project Description:

A. Study of diseases in man

1. Mycosis fungoides

Thirteen patients have expired and have been autopsied. Dr. J. Lasersohn, Resident in Pathologic Anatomy Section, is preparing a description of the morphologic changes in those five patients who have developed cardiac arrhythmias and cardiac decompensation associated with the presence at necropsy of tumors in the myocardium. Dr. A. Engel, Dr. J. Block, and I are writing a clinico-pathological essay on the course of the disease in those patients who have died.

2. Melanomas

No progess has been made in studying human naevi and melanomas.

B. Study of diseases in animals

1. A description of the malignant melanotic tumors produced in guinea pigs by cutaneous application of dimethylbenzanthracene is to be submitted for presentation at the A.A.C.R. meeting in April 1961. One of the melanomas grew and metastasized on transplantation to one guinea pig. Whether this tumor will continue to grow on transplantation is not at present known.

2. The study of hair growth in chinchillas continues.

Part A (continued)

C. Miscellaneous collaborations and efforts

1. With Dr. R. Love, formerly NCI.

Dr. Love and I have been studying C3H mice 18 months of age who were injected at birth with SE polyoma virus. The experiment has been terminated, and Dr. Love now has the material.

2. With Dr. R. Hertz, NCI, and Drs. W. Ober and E. Price

A description of the pathologic findings in patients with choriocarcinoma is being prepared.

3. With Dr. R. Hertz, NCI.

A description of twenty-two necropsies of patients who died with carcinoma of the adrenal cortex is being prepared.

Significance: It is my work to diagnose cancer and to demonstrate the pathogenesis of disease in patients. My experimental work is chiefly concerned with the histogenesis of melanomas and the factors involved in their development.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Tobie, John E., Edgcomb, John H., and Freireich, E. J.
Tongue Worm (Linguatula Serrata) infestation in a patient with acute leukemia
Am. J. of Clin. Path. 28: 628-633, 1957

2. Utz, J. P., Van Scott, E., Bernton, H. W., Edgcomb, J. H., Bunim, J. J., Bell, N. H., and Kaufman, H. E.
Sarcoidosis: Clinical staff conference at the National Institutes of Health
Ann. Internal Med. 51: 1356-1370, 1959

3. Merchant, R. K., Louria, D. B., Geisler, P. H., Edgcomb, J. H., and Utz, J. P.
Fungal Endocarditis: Review of the literature and report of three cases
Ann. Internal Med. 48: 242-266, 1958.

4. Edgcomb, John H.
On the development of peptic ulcers in patients treated with prednisone or prednisolone
Schw. Zeit. fur Allgemeine Path. und Bakt. 21: 363-372, 1958.

Honors and Awards relating to this project:

Lecturer in Pathology, Howard University
Member of a mission of Government pathologists to U.S.S.R.

Serial No. NCI 525

PHS-NIH
Individual Project Report
Calendar Year 1960

1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda 14, Maryland

Part A.

Project Title: Attempt to induct carcinoma of the bladder in hamsters by infection with Schistosoma haematobium.

Principal Investigator: Dr. Eloise Cram.

Other Investigators: Dr. Louis B. Thomas, Dr. Elmer Berry and Dr. Louis Olivier.

Man Years (calendar year 1960):
Total: 1
Professional: 1
Other: -

Patient Days (calendar year 1960):

Project Description:

This research project is in collaboration with Doctors Eloise Cram, Elmer Berry and Louis Olivier, N.I.A.I.D. and consists of studying chronic infection with S. haematobium (Gold Coast strain and Egyptian strain) in hamsters; particularly with reference to the induction of bladder carcinoma.

a.) 217 hamsters were exposed to approximately 300 cercariae (Gold Coast strain S. haematobium) each early in 1955. All of these hamsters have now died and have been autopsied. Histological sections have been prepared and are being studied microscopically.

b.) Late in 1956 and during the first six months of 1957, S. haematobium from Egypt became available through the efforts of Drs. Berry and Olivier. Approximately 150 hamsters were exposed to cercariae. All of these animals have now died and microscopic sections have been prepared for study.

Thus when this experiment is completed it will be possible to compare chronic S. haematobium infestation in the two groups of animals with Egyptian and Gold Coast strains of S. haematobium.

Significance to Cancer Research:

S. haematobium infection has been found associated with bladder carcinoma in several parts of the world. This study is designed to produce bladder cancer in the hamster by chronic infection with S. haematobium. The incidence of bladder cancer associated with Schistosomiasis is high in Egypt and relatively low in the Gold Coast. The study is also designed to determine whether there are pathological differences between infestation due to S. haematobium from these two parts of Africa.

Part A (continued)

Proposed Course of the Project:

Grossly, no bladder tumors have been observed. During the next year histologic sections of the autopsied animals will be studied so that this experiment can be completed.

Part B included Yes ( ) No (x)

Serial No. NCI 528
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Cytochemistry of Ribonucleoproteins

Principal Investigator: Robert Love

Other Investigators: R. Gerald Suskind (A,B)
N. P. Salzman (D)
Alan S. Rabson (C)
Mr. R. H. Liles (E)

Cooperating Units:

Man Years (calendar year 1960):
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To study ribonucleic acid metabolism of normal, neoplastic and virus-infected cells.

Methods: A. Further development of new staining procedures for ribonucleoproteins.

B. Cytochemical studies of HeLa and monkey kidney cells infected with a new (probably RNA) virus, parainfluenza #3.

C. Further cytochemical studies of $P388D_1$ cells grown in milk medium and infected with polyoma virus.

D. Cytochemical and biochemical investigation of the nucleoproteins of HeLa cells in which DNA synthesis is blocked by 5-fluorodeoxyuridine (FUDR).

E. Investigation of the nature and properties of a ribonuclease contaminant of bovine testicular and streptococcal hyaluronidase.

Major Findings: A. (1) Nine types of ribonucleoproteins which differ in morphology, staining properties and functional behavior have been characterized.

Part A (continued)

(2) A new staining procedure has been developed in which the nucleolus can be stained metachromatically and all other structures in the nucleus are colored green. Studies are in progress, using this method, to determine the site or sites of formation of nucleolar material in telophase.

(3) In view of the deposition of electron-dense molybdate at sites of RNA in cells stained by the toluidine blue-molybdate procedure, material has been prepared for electron microscopy. This material has been given to Drs. A. J. Dalton and W. Gaylord but has not been examined.

B. In HeLa cells infected with parainfluenza virus, syncytial formation does not appear to be related to the production of infectious virus and may appear before or after the release of virus into the tissue culture fluid. "Inclusions" are formed in the syncytia by degeneration of nuclei. Intranuclear and cytoplasmic ribonucleoprotein inclusions are formed in monkey kidney cells infected with parainfluenza virus; neither the presence of inclusions nor the formation of virus seems to interfere with cell division. There is evidence for the production of an interferon-like substance in HeLa cells infected with parainfluenza virus.

C. By means of improved techniques and more frequent and more intensive analysis of $P388D_1$ cells infected with polyoma virus abnormal DNA has been demonstrated in the infected cells and alterations in the ribonucleoproteins have been elucidated further. Studies are in progress to relate the changes in nucleoproteins to synthesis and release of virus.

D. Preliminary results indicate that when DNA synthesis is inhibited by FUDR cell division is prevented, nuclear RNA synthesis is inhibited and cytoplasmic RNA synthesis is unaffected. After exposure to FUDR for 24 hours RNA associated with the chromatin decreases, the nucleolus and nucleolinus are increased in size and cytoplasmic RNA is increased. Studies of the effects of FUDR on the nine types of ribonucleoprotein demonstrable in the cell are in progress.

E. Methods have been devised for the inhibition of the ribonuclease component which is present in all preparations of hyaluronidase and which has led to erroneous interpretation of the effects of hyaluronidase.

Significance: The role of RNA metabolism of the cell is of fundamental importance in the understanding of genetics, mutation, protein synthesis, cell growth and differentiation, neoplasia and virus infection.

Part A (continued)

Proposed Course: No further work will be done at N.I.H. A grant of $440,827 has been awarded to assist in the continuation of this work at the Jefferson Medical College, Philadelphia, on a much larger scale than is possible at N.I.H. Furthermore, the payment of much larger salaries to the investigators provides additional encouragement for transferring the research elsewhere.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Love, R., and Rabson, A. S.
Cytochemistry of nucleic acids in murine lymphoma cells infected with polyoma virus in vitro.
Nature. In press.

2. Love, R., Liles, R. H., and Ellem, K. A. O.
Activity of ribonuclease in preparations of testicular and streptococcal hyaluronidase.
J. Histochem. & Cytochem. In press.

3. Love, R., and Rabson, A. S.
Nucleoproteins in murine lymphoma cells infected with polyoma virus in vitro.
Pathologie et Biologie. In press.

4. Love, R., and Suskind, R. G.
Further observations on the nucleoproteins of mitotically dividing mammalian cells.
Exper. Cell Res. In press.

5. Love, R., and Suskind, R. G.
Characterization of nine types of ribonucleoproteins in the cell.
Ann. Histochimie. In press

Serial No. NCI 529
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Viral Therapy of Tumors

Principal Investigator: Robert Love

Other Investigator: R. Gerald Suskind

Cooperating Units:

Man Years (calendar year 1960):
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To adapt viruses to become oncolytic for tumors.

Methods: A. Passage of Newcastle Disease Virus (NDV) through various tumors of mice.

B. Analysis of the effect of NDV that is oncolytic for the Ehrlich ascites tumor on spontaneous mammary tumors of mice.

Major Findings: A. (1) NDV isolated from chickens and passed in the chick embryo could not be adapted to become oncolytic by repeated passage through the Ehrlich or the Krebs ascites tumors in vivo. Virus multiplication did not occur.

(2) The same strain of virus grew to some extent in P388 ascites cells in vivo and after alternate passages in the tumor and in eggs produced some oncolysis. It has not yet been possible to adapt this virus to produce oncolysis regularly in P388 cells in vivo.

(3) Adaptation of NDV to become oncolytic for the Ehrlich tumor made it more easily adaptable to the Krebs tumor than the original chicken virus but made it less readily adaptable to the P388 lymphoma.

B. Inoculation of mice bearing large spontaneous mammary tumors with undiluted or a 10^{-1} dilution of NDV that produced complete regression of the Ehrlich tumor killed the mice. Neither oncolysis nor viral growth in the tumor could be demonstrated.

Part A (continued)

Significance: The present approach to the treatment of spontaneous neoplasia with viruses is not very hopeful. Other methods, such as the use of infectious nucleic acid and the production of viral mutants by chemical or other means will be necessary for the creation of oncolytic strains of virus.

Proposed Course: This work will be continued on a small scale at the Jefferson Medical College.

Part B included: No

Serial No. NCI 536
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: A review of the surgical and autopsy sections from veterans of World War I whose cause of death has been listed as lung cancer

Principal Investigator: Katherine McD. Herrold

Other Investigators: Harold L. Stewart, Laboratory of Pathology, NCI,
Harold F. Dorn, Biometry Branch, DRS, NIH

Cooperating Units:

Man Years (calendar year 1960):
Total: 1/3
Professional: 1/3
Other: --

Project Description:

Objectives: The objective of the study is to review all the cases from veterans of World War I which have been diagnosed as lung cancer and to determine whether the findings meet the criteria for primary lung cancer. The second objective is to classify the tumor according to the histological type.

Methods: This review is being done in conjunction with the epidemiological study of the National Cancer Institute on the relationship between smoking and death from carcinoma of the lung.

Major Findings: (1) The diagnosis of primary lung cancer in many cases has been based only on the clinical and roentgenological findings.

(2) A certain percent of the undifferentiated carcinomas are being classified as anaplastic adenocarcinoma with the use of special stains for mucin.

Significance: It has been proposed that the ratio of Group I to Group II tumors may be a sensitive index of a difference in the incidence of environmentally caused lung cancer between two or more population groups.

Part A (continued)

Proposed Course: To continue reviewing the surgical and autopsy sections from the cases of veterans of World War I whose cause of death has been listed as lung cancer. To determine if there is a correlation between the smoking histories and histological type of primary lung cancer.

Part B included: No

Serial No. NCI 536a
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Experimental studies relating to the minimal concentration of certain polycyclic hydrocarbons which will produce cancer in hamster cheek pouches

Principal Investigator: Katherine McD. Herrold

Other Investigators:

Man Years (calendar year 1960):
Total: 1/3
Professional: 1/3
Other: --

Project Description:

Objectives: The objective of the study is to determine the minimal concentrations of known carcinogens which will produce cancer in hamster cheek pouches and to study the histologic changes preceding the development of invasive carcinoma.

Methods: Test pellets using C.P. beeswax as a vehicle and containing various concentrations of 20-methylcholanthrene and 9,10,dimethyl-1,2, benzanthracene were prepared. The pellets were inserted in the right cheek pouch of 83 hamsters and were left in situ for indefinite periods. Pellets consisting only of beeswax have been inserted into the cheek pouch of male and female hamsters and will serve as controls. The technique used is the one developed by Dr. Lucia Dunham (see NCI 501a).

Major Findings: A marked difference has been observed between the action of 20-methylcholanthrene and 9,10,dimethyl-1,2,benzanthracene on the mucous membranes of the hamster cheek pouches. Concentration of 2.5 mgms. or more of 9,10,dimethyl-1,2,benzanthracene produce acute inflammation, ulceration and necrosis of the hamster cheek pouch and within a period of 10 days to two weeks the pellet sloughs out. The end stages in many of these pouches is atrophy and in a few of the cases neoplastic changes have occurred. With lower concentration of 9,10,dimethyl-1,2,benzanthracene, epidermoid carcinoma has developed in many of the cheek pouches. The livers of the tumor-bearing animals reveal prominent extramedullary hematopoiesis.

Part A (continued)

Significance: High concentrations of 20-methylcholanthrene have for the most part produced only negligible changes in the hamster cheek pouch. Two tumors have developed. The control animals reveal no significant pathological changes in the pouches. Liver cysts, ovarian cysts, amyloidosis involving liver, spleen, and kidney, adrenal cortical adenomas, adenomatous polyps of intestinal tract and malignant lymphomas have been frequently encountered.

Proposed Course: A report is being prepared at the present time for publication. The variable response to the polycyclic hydrocarbons studied would seem to indicate that this technique would not be a sensitive enough method for testing substances suspected of being weak carcinogens.

Part B included: No

Serial No. NCI 536b
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Experimental studies relating to the production of lung tumors in Syrian hamsters. Study of the development of metastases in Syrian hamsters with tumors induced by different methods

Principal Investigator: Katherine McD. Herrold

Other Investigators: Dr. Elizabeth Chu

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 2/3
Other: --

Project Description:

Objectives: The objective of the study is to attempt to produce lung tumors in Syrian hamsters. To compare the pattern of metastases of different tumors and to determine if there is any difference in the predilection of these tumors for certain organs.

Methods: Syrian hamsters used as experimental animal.
1. Endotracheal instillation of polyoma virus in milk culture medium.
2. Weekly endotracheal application of 3,4 benzpyrene to the tracheo-bronchial tree.
3. Implantation of thorium wire into the bronchi.
4. Cytosieve technique for study of metastatic pattern of different tumors.

Major Findings: 1. Lung tumors have developed with the polyoma virus and are transplantable.
2. Epithelial atypism of the bronchial epithelium and a squamous cell papilloma has been induced with weekly applications of 3,4 benzpyrene.
3. Acute lobular pneumonia has been the cause of early death in many of the thorium treated animals. No tumors have so far been produced.
4. The pattern of the metastatic spread of three different tumors is being studied. One tumor has been induced by the polyoma virus; one by a polycyclic hydrocarbon and the other tumor arose spontaneously in a Syrian hamster.

Part A (continued)

Significance: 1) To determine whether lung tumors similar to human lung carcinomas can be produced in the Syrian hamster with 3,4 benzpyrene and thorium wire.

2) To study factors which influence metastases of hamster tumors.

Proposed Course: To continue the experimental studies as listed under methods above.

Part B included: No

Serial No. NCI 503
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Collaborative research requiring Pathologic Anatomy

Principal Investigator: Thelma B. Dunn

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To collaborate with and advise other investigators at the National Cancer Institute in phases of research problems requiring experience in animal pathology.

Methods: This may consist of (1) full responsibility and separate publication as senior author of material reviewed in connection with work of another investigator, (2) review and description of animals coming to autopsy, and publication of the findings as a separate section of a paper describing the entire experiment, (3) review and diagnosis of tumors found by investigator in course of experiments, the information to be used in a tabulation of results, (4) occasional consultation and advice on pathologic aspects or material in an experiment.

Major Findings: (1) With Dr. Ruth Merwin. Work is continuing on the effect of introducing semipermeable membranes and other substances into the peritoneal cavity of inbred mice. The BALB/c mouse reacted by producing intraperitoneal plasma cell neoplasms, which are transplantable and have characteristic protein electrophoretic patterns resembling multiple myeloma in man. These are now being studied by many investigators, both at NIH and elsewhere. The reaction of the C3H mouse to the same substances does not produce plasma cell neoplasm, but severe fibrosis and occasional ossification. The results are not yet tabulated, but a study of the histogenesis of the fibrosis and the plasma cell neoplasms is in progress.

Work has also been done with these tumors by Dr. Kobayashi, a Visiting Scientist. A report of his work is attached (NCI 503a).

Part A (continued)

(2) With Dr. Katherine Sanford. The morphology of tumors grown in the mouse from tissue culture cells. (Reports to be made by Dr. Sanford.)

(3) Work with Dr. Andervont on tumors of wild mice. Histology of the tumors is similar to tumors found in inbred mice.

Significance: Pathologic review is indispensable to many types of cancer research. This is best carried out by a specialist in pathologic anatomy, since with the present complexities of research a single investigator cannot know this field and his own specialty.

Proposed Course: This work will continue as in the past. When, as a result of many observations, enough information is accumulated regarding any subject, publications will be made.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Dunn, T. B., Potter, M., Fahey, J. L., and Merwin, R. M. Morphology and serum protein changes in plasma cell neoplasms in mice. Arch. de Vecchi 31: 67-77, 1960.

Honors and Awards relating to this project:

Council Member, Washington Society of Pathologists
Vice-President and President-elect, American Association for Cancer Research

Serial No. NCI 503a
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Study of the reaction of bone to plasma cell and other neoplasms in mice.

Principal Investigator: Hiroshi Kobayashi

Other Investigators: Thelma B. Dunn, Michael Potter, Laboratory of Biology

Cooperating Units:

Man Years (calendar year 1960):
Total:
Professional:
Other:

Project Description:

Objectives: To investigate the osteolytic lesions developing from intramedullary growth of plasma cell neoplasms, and to compare with the intramedullary growth of other tumors.

Methods: Neoplastic plasma cells from ascites tumors were injected intravenously, and growth within the bone marrow resulted. Bits of tissue were taken directly from solid plasma cell tumors and placed within the marrow cavity, or directly on the peritoneal surfaces. Other neoplasms were treated in similar fashion. Studies were made by use of papain cleared skeletons, and histologic sections.

Major Findings: The plasma cell neoplasms produced both osteolytic and osteoplastic reactions. A study of the effects of other neoplasms is not completed.

Significance: The mechanism by which the osteolytic reaction of neoplastic plasma cells is produced is not understood. This study attempts to explain this, and makes comparisons with multiply myeloma in man.

Proposed Course: Enough work has been completed for a paper now in preparation. The principal investigator will soon return to Japan but it is hoped he can continue this type of work.

Serial No. NCI 503b
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Editing of translation of book by W. Fischer and I. Kühl on tumors of laboratory rodents

Principal Investigator: Thelma B. Dunn

Other Investigator: Dr. Renate Kimbrough

Cooperating Units:

Man Years (calendar year 1960):
Total: None as yet from NCI
Professional:
Other:

Project Description:

Objectives: This book in German is a valuable reference containing a very complete review of the European literature. It is desirable that it be made available in English.

Methods: A contract was awarded to Dr. Renate Kimbrough to make a literal translation from the German to English. It was also arranged that Dr. I. Kühl would revise certain parts of the book and bring it up-to-date.

Major Findings: The translation has been completed by Dr. Kimbrough although translation of some of the revisions awaits material to be received from Dr. Kühl.

Significance: No satisfactory reference book on this subject is now available and one is badly needed.

Proposed Course: Editing is yet to be done. When this is completed it is proposed to publish it as a monograph in the Journal of the National Cancer Institute.

Part B included: No

Serial No. NCI 504
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: An electron microscopic investigation of normal and pathologic tissues

Principal Investigator: William G. Banfield

Other Investigators: Darlene C. Brindley, Clyde J. Dawe, Seymour Sabesin, W. P. Rowe, Eugene Van Scott

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: 1. To describe and interpret the ultrastructure of selected pathologic states and to compare the results with suitable control tissues.

2. To look for and study the inciting agent or accompanying virus in neoplastic and other disease states using human, animal, and tissue culture material.

Major Findings: 1. In some of the basal cells of normal skin epidermis very denze granules 50 A° in diameter with the structure of ferritin are found. However, these granules have not been present in all normal skin. In hemachromatosis they occur throughout the malpighian layer.

In collaboration with Dr. Eugene Van Scott psoriatic epidermis has been examined. Here there is intercellular edema and defects in keratohyalin and keratin formation. The keratohyalin granules are few, small, and more regular than normal. They may develop a membrane and some persist into the keratinized layer. Often in the lower layers of the corneum the condensed cells contain compact cytoplasmic elements. So far ferritin has not been seen in psoriatic epithelium.

2. In collaboration with Dr. Clyde J. Dawe we have demonstrated that virus particles can be found easily in polyoma induced tumors kept in a maintenance media in tissue culture for as little as 24 hours. Virus is not found after a reasonable amount of searching in

Part A (continued)

tumors examined directly. Several human tumors were held in tissue culture from 24 hours to two weeks without uncovering anything suggestive of an agent when looked at with the electron microscope.

3. In collaboration with Drs. Rowe and Dawe we have obtained pictures of the mouse "adenovirus" which morphologically place it with the adenoviruses.

4. In collaboration with Dr. W. P. Rowe we have identified the mouse thymic agent which has features in common with the Lucke, salivary gland, herpes simplex, and herpes B viruses.

Significance: 1. Ferritin has not previously been reported in the skin epithelium nor have there been previous electron microscopic studies of psoriasis.

2. An easy method of uncovering polyoma in induced hamster tumors is demonstrated but it does not seem to work with the human tumors so far examined.

3. Two new animal viruses have been seen for the first time.

Proposed Course: 1. Montages from a number of cases of psoriasis using the psoriatic lesion and uninvolved skin and montages from normal skin will be prepared and compared.

2. Selected tumors both animal and human will be examined both directly and from tissue culture as well as promising material from any source for new viral forms.

Part B included: No

Serial No. NCI 504a
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: An investigation of the collagenous connective tissues and elastin

Principal Investigator: William G. Banfield

Other Investigators: Dr. Sokoloff, Dr. Obriend, Dr. Sabesin, Dr. White, Dr. Ried, Dr. Jackson, Darlene C. Brindley

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: 1. To elucidate age changes and pathologic alterations in collagen and elastin.

2. To uncover mechanisms affecting the state of collagen in the body.

Major Findings: 1. The relative amounts of acetic acid soluble collagen have been measured in back and abdominal skin of hamsters from 28 to 1004 days of age. There is less soluble collagen in the back skin as compared to the abdominal skin. Except for a rise in the soluble collagen in the abdominal skin between 28 and 42 days there is a reduction of the amount of soluble skin collagen with increasing age. The age pattern of solubility is sufficiently clear-cut to allow for testing the effects of any factor on this aspect of the collagen metabolism in hamsters in a wide range of ages.

A correlation of histologic changes in body organs with the collagen changes has been attempted. If correlations exist they will be complex and difficult to uncover.

2. As an outgrowth of the studies on aging hamsters we have 16 transplantable tumors among which are 2 melanomas, one adrenal cortical tumor, one myxoma, one epidermoid carcinoma, one adenocarcinoma of the bowel and two reticulum cell sarcomas.

Part A (continued)

3. With Drs. Sokoloff and Obrien we have studied achronotic joint cartilage both pigmented and unpigmented. The pigment was not identifiable in the electron microscope. In one instance marked changes in the collagen fibrils were present in the pigmented cartilage evidenced by increased width, ragged margins and collagen fibrils swollen into amorphous structures with loss of striation.

4. Dr. Sabesin has produced the Arthus reaction in rabbits and guinea pigs using ferritin. He has begun to examine these lesions in the electron microscope. The studies on the collagen from the foot of a patient with rheumatoid arthritis was not productive.

5. A pilot experiment has been completed in collaboration with Drs. White, Ried, and David Jackson for the purpose of comparing the rate of uptake of C^{14} labeled diet incorporated glycine in the abdominal skin, back skin, tail tendon, and Achilles tendon of the rat. When started at conception there was very little uptake by the time of weaning. One week after weaning the uptake increased markedly with the slowest uptake in the tail tendon.

6. With the aid of Mr. G. Peckham (summer student) approximately 30 skin samples from individuals over 60 years of age were collected for comparing the dermal collagen fibril widths with those from younger age groups.

Significance: 1. A useful tool for ascertaining an effect of any factor on collagen metabolism as expressed by the acetic acid solubility is now available in the hamster.

2. Several spontaneous transplantable hamster tumors are available for study.

3. The case of ochronosis is one of the few instances where a pathologic change in collagen fibrils has been demonstrated. A previous report of visualization of ochronotic pigment using kidney could not be confirmed using cartilage.

Proposed Course: 1. The large amount of data we have collected for the normal aging hamster will be subjected to statistical analysis and normal weights etc., will be published. Further attempts at correlating this data with the collagen solubility data, together with any other correlations which may seem profitable will be made.

2. The collection of spontaneous hamster tumors will be studied in more detail.

Part A (continued)

3. Dr. Sabesin will continue his study trying to trace the ferritin particle as it elicits the Arthus reaction.

4. The main experiment using C^{14} labeled glycine to study differences in metabolism in different connective tissues of the rat has been underway for three months. After eight months to one year the C^{14} in the diet will be stopped and the rate of decay in the tail tendon, Achilles tendon, back and abdominal skin will be followed.

5. Dermal collagen fibril widths will be measured from groups of individuals representing a wide range of ages.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. O'Brien, W. M., Banfield, W. G., and Sokoloff, L.
Studies on the pathogenesis of ochronotic Arthropology
Arthritis & Rheumatism 4. 1961. In press.

Serial No. NCI 504b
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Contagious tumors

Principal Investigator: William G. Banfield

Other Investigators: Darlene C. Brindley, Graciela Ramirez

Cooperating Units:

Man Years (calendar year 1960):
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To study a contagious hamster tumor, attempt to find the mode of contagion, and to discover similar tumors.

Major Findings: 1. A spontaneous reticulum cell sarcoma of the hamster was successfully carried through 12 passages. Generalized cutaneous involvement was present in the first and second passages. Thereafter the growth was typified by widespread metastases and a large ulcerating tumor at the injection site, with local skin invasion only. In the terminal phases of transplanted growth, the peripheral blood became flooded with tumor cells. Injections of blood, ascites fluid, and brain from tumor-bearers produced tumor in the host. Transfer of cell-free filtrates of brain, ascites fluid, and tumor have not produced tumor.

Ten untreated animals were caged with ten animals inoculated with the tumor. Of the untreated animals, six died with laryngeal obstruction caused by submucosal tumor growth. Two were killed, with the generalized systemic involvement similar to that caused by solid tumor inoculations.

Fresh tumor tissue was fed to six separated animals. One developed the systemic tumor involvement, another the laryngeal occlusion only.

The tumor arose in one of three hamsters separated by a screen from tumor-bearing animals.

Dr. Ramirez has been examining this tumor with the electron microscope but so far no agent has been recognized.

Part A (continued)

Significance: Outside of the venereal tumor of the dog, visceral lymphomatosis in chickens and the Rous sarcoma, this is the only other tumor transmitted by contact.

Proposed Course: 1. Continue to search for the mode of transmission.

2. In collaboration with Dr. O'Conor attempt to obtain specimens of a similar appearing tumor in Africa and study it using the electron microscope and in collaboration with others using tissue culture, transplants, etc.

Part B included: No

Serial No. NCI 505
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The induction of squamous carcinoma of the lungs in laboratory animals

Principal Investigator: Mearl F. Stanton

Other Investigators: Robert Blackwell, Joseph Albrecht and staff

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To induce lung cancer in laboratory animals through deposition of known and suspected carcinogens in areas of regenerating lung tissue.

Methods: Certain aliphatic halocarbons are capable of causing ishemic necrosis when trapped in capillary vessels. By incorporating carcinogens such as methylcholanthrene into the inoculum it is possible to deposit the carcinogen in particular organs of interest to the oncologist. The detection of methylcholanthrene deposition by U.V. microscopy is being accomplished.

Major Findings: Intravenous administration of the halocarbon alone has not induced tumors in rabbits, rats, or mice, many under observation for one to two years. In mice and rabbits neither the halocarbon nor halocarbon + methylcholanthrene cause an increase in spontaneous alveologenic tumors nor do they induce carcinomas in the areas of infarction. In rats a suspension of methylcholanthrene in tricaproylin carried to the lungs in the halocarbon consistently induced metastasizing squamous carcinomas of the lung which kill the animals in six to seven months. The histogenesis of the tumors now under study indicates they arose in areas of bronchial metaplasia which result from halocarbon produced infarction. We have traced the deposition of methylcholanthrene with ultraviolet light microscopy.

Part A (continued)

Significance: Our studies may contribute to several major problems in the genesis of lung cancer in man such as the role of pulmonary scarring and its relationship to exogenous inhaled carcinogens which in themselves may act by injuring bronchial cells and subsequently altering their neoplastic potential. Such studies may also offer a new method for studying the mechanism of methylcholanthrene carcinogenesis.

Proposed Course: Study of the histogenesis of the tumors. Quantitative measurements of dose to response. Further testing on ability of scarring alone to induce carcinoma. Tests currently under study include the intravenous administration of infarct inducing halocarbons several days prior to and several days after intravenous methylcholanthrene in an effort to demonstrate the initiating and/or promoting effects of these substances. Further studies as outlined in Individual Project Report NCI 505, 1957, are also continuing.

Part B included: No

Serial No. NCI 506
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Morphologic aspects of the Moloney leukemia virus

Principal Investigator: Thelma B. Dunn

Other Investigator: John B. Moloney

Cooperating Units:

Man Years (calendar year 1960):
Total: 7/12
Professional: 1/4
Other: 1/3

Project Description:

Objectives: The Moloney virus has furnished an opportunity to study the pathogenesis of leukemia in mice and rats.

Methods: Control and virus infected mice were killed at frequent intervals, and the histogenesis of leukemia was followed. A similar study has been completed for rats, but the microscopic slides are not yet reviewed. Some additional evidence on the action of the virus has been obtained from mice that were thymectomized or splenectomized, or treated by irradiation.

Major Findings: Alterations in reticulo-endothelial organs precede the development of leukemia. These alterations are particularly notable in the thymus and spleen.

Significance: The Moloney virus is almost ideal for studies of the pathogenesis of leukemia, since it is effective in fully grown animals, and in almost 100 percent of treated animals.

Proposed Course: A paper on the morphology of the virus induced leukemia in mice is now in press. This was a basic study, carried out in detail. It is anticipated that as much time as possible will be spent on this study since the material is readily available and it is an important lead in present studies of viruses and leukemia.

Part B included: No

Serial No. NCI 507
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Factors involved in abortive mitosis in human hair follicles in the catagen (resting) phase of the growth cycle

Principal Investigator: Ross C. MacCardle

Other Investigators: E. J. van Scott

Cooperating Units:

Man Years (calendar year 1960):
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objectives: We discovered that the epithelial cells of human hair follicles in the resting (catagen) phase of the hair growth cycle reveal evidence of arrested mitotic figures, whereas in the actively growing phase few if any such abortive mitotic figures could be found.

Methods: A microscopic study of biopsy specimens.

Significance: We are interested in determining whether basal and squamous cell tumors possess such figures. We are also interested in trying to determine whether some naturally occurring mitotic inhibitor may be present during the catagen phase and neutralized in the anagen phase,--a situation similar to the production of acetylcholine and its breakdown by cholinesterase. It was found that human cutaneous basal-cell tumors possess many arrested mitotic figures while squamous-cell tumors possess none of them. It would be of interest to determine whether the benign basal-cell tumors possess an inhibitor of mitosis which may be blocked upon transformation to squamous-cell tumors.

Proposed Course: Finishing it for final proof, and we plan to attempt to extract a chemical agent from the catagen stage that would throw anagen into catagen.

Part B included: No

Serial No. NCI 508
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Cytology of antibody-producing plasma-cell tumors of mice and human multiple myeloma

Principal Investigator: Ross C. MacCardle

Other Investigators: Michael Potter and John L. Fahey

Cooperating Units:

Man Years (calendar year 1960):
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objectives: To determine the intracellular site of production of antibodies and to ascertain the cellular origin and fate of plasma cells.

Methods: By cytological techniques for Golgi apparatus mitochondria and RNA, we study the tumor tissue and also plasma cells induced to form in mice injected with an adjuvant plus staphylococcus.

Major Findings: The juxtanuclear clear cytoplasmic 'hof' by which the plasma cell is characterized consists of the Golgi apparatus and the centrosome. The plasma-cell tumors of mice possess three different types of Golgi apparatus consisting of: I. a few small strands; II. a diffuse network; and III. a large thick hypertrophied ball network. Two of the tumors producing beta-globulin can be distinguished from the others by their characteristic hypertrophied Golgi apparatus (Type III). However, the type of Golgi apparatus does not appear to be related to the electrophoretic mobility of the globulin, but rather to the speed of production of the protein. The cytoplasm of plasma cells is rich in RNA, but the Golgi area is free of it.

The plasma cells of these tumors appear to derive from undifferentiated mesenchymal cells around blood vascular capillaries and adipose cells of the intestinal mesenteries and parietal peritoneum. Mitotic division occurs in the undifferentiated cells before the juxtanuclear hof appears. The maturing plasma cells evidently secrete globulins of different electrophoretic mobility at different stages of differentiation. These globulins do not behave as antibodies, but it is believed that they may be immature antibodies.

Part A (continued)

It was found that these tumors develop chiefly around blood vessels. After transplantation into hosts, they grow in fascicles in the interstices of a blood vascular network of the transplant. Normal plasma cells, which are smaller and less differentiated, develop in the same way following intraperitoneal injection of silica or mineral oil.

Significance: Such information may lead to better understanding of the mechanism of production of antibodies and to a method of controlling the differentiation of plasma cells in plasma-cell tumors and multiple myeloma.

Proposed Course: We intend to pursue this problem in an attempt to prove further the cellular origin of plasma cells. A manuscript is being prepared for publication, describing the histological characteristics of these different tumors in respect to the globulins produced. This work was presented at the International Congress of Cell Biology in Paris.

Part B included: No

Serial No. NCI 509
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The protoplasm of tumor cells: Characteristics of malignant neoplastic protoplasm

Principal Investigator: Ross C. MacCardle

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/8
Other: 7/12

Project Description:

Objectives: This manuscript consists of about 300 typewritten pages being written in monograph form as part of an International Handbuch der Protoplasmaforschung edited by Heilbrunn and Weber. It describes the cytological and some physiological characteristics of tumor cells. It contains some new material such as plasma-cell tumors and pituitary tumors that has not been published elsewhere.

Proposed Course: To be sent to the printers in January. This will finish the work.

Part B included: No

Serial No. NCI 510
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Study of endocrine and reproductive organs in the mouse

Principal Investigator: Thelma B. Dunn

Other Investigators: Dr. Sam Spicer on structures found in the adrenal gland

Cooperating Units:

Man Years (calendar year 1960):
Total: 5/6
Professional: 1/3
Other: 1/2

Project Description:

Objectives: This is a continuation of work begun several years ago. It is expected that it will continue for a considerable period of time since time is required for accumulation of animals. To accumulate reliable data by observations on untreated mice, planned procedures, and reviews of the literature regarding the reproduction and endocrine organs of inbred mice. During the past year work has been concentrated on the adrenal gland.

Methods: Personal observations on material submitted from different sources, planned experimental procedures in inbred mice, histologic study of untreated mice at various ages, systematic review of the literature.

Major Findings: This study is proceeding slowly because of pressure of other work. Considerable morphologic difference is found in the adrenal glands of inbred strains of mice. This material is already proving useful to other members of the staff since it represents a normal standard of some endocrine organs in the mouse. A beginning has been made on writing up results on the adrenal gland. The literature, which proved unexpectedly voluminous, has been reviewed. Amorphous particles seen in the medulla in Zenker-formol fixed adrenals are being studied by Dr. Sam Spicer. He has found similar particles in the adrenal medulla of other species. Mercuric chloride is required to precipitate them and no previous description has been found of these bodies.

Part A (continued)

Significance: Since hormonal studies are an important part of cancer research, it is desirable that reliable information be available regarding the endocrine and reproductive organs of inbred strains of mice.

Proposed Course: It is expected that this study will proceed slowly, and require several years for completion.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Spicer, S., and Dunn, T. B.
Reducing bodies in the adrenal medulla
J. Nat. Cancer Inst. In press.

Serial No. NCI 511
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Collaborative research

Principal Investigator: Richard L. Swarm

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: To collaborate with and/or advise other investigators in phases of research requiring experience in pathologic anatomy or the use of radioactive isotopes.

Methods: Methods vary widely and are dependent upon the problem and the extent of the collaboration.

Major Findings: Collaborative research includes the following projects:

(1) Collaboration with Dr. Harold Morris continues as noted earlier. New collaboration by Dr. Morris and Dr. Dyer in the rats with congenital hyperbilirubinemia or jaundice studies earlier by Dr. Swarm with Dr. Schmid of NIAMD has followed the detection of different patterns of conjugation of certain hepatic carcinogens in these animals. These animals with a genetic defect, an inability to conjugate bilirubin with glucuronic acid are unable to conjugate certain carcinogens with glucuronic acid. A preliminary study of carcinogenesis in these animals shows that carcinogenesis occurs in these animals as it does in non-jaundiced littermate controls.

(2) Work reported with Dr. Spicer in last year's report is in press in Laboratory Investigation.

(3) Study with Dr. G. Sharp and Dr. F. Smith of thyroid carcinoma growth in animals immunized with rat thyroglobulin and crude thyroid carcinoma extract in adjuvant has been begun. Early results show a delay in the appearance and slower growth of tumor in rats immunized

Part A (continued)

with rat thyroglobulin in adjuvant. Morphological studies to detect any alterations present in the tumor in immune hosts are in progress. (This work is of interest and related to project #512.)

(4) As a result of past collaboration, interest continues in the distribution of tetracyclines in necrotic tissue and certain neoplasms.

(5) During the past year Dr. Swarm has consulted with a few other scientists about their research work and has lectured in four NIH Isotope Training Courses given during the past year.

Significance: Collaboration is indispensable to many types of cancer research.

Proposed Course: Collaborations listed will be continued as desired.

Part B included: No

Part A (continued)

with rat thyroglobulin in adjuvant. Morphological studies to detect any alterations present in the tumor in immune hosts are in progress. (This work is of interest and related to project #512.)

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(5) During the past year Dr. Swarm has consulted with a few other scientists about their research work and has lectured in four NIH Isotope Training Courses given during the past year.

Significance: Collaboration is indispensable to many types of cancer research.

Proposed Course: Collaborations listed will be continued as desired.

Part B included: No

Serial No. NCI 512
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Transplantation studies

Principal Investigator: Richard L. Swarm

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: To study the survival and alteration of isografts and homografts of cartilage, other normal tissues and tumor tissue and to determine the pattern of host reaction to these grafts.

Methods: The environment of cartilage and other tissue grafts is altered by transplantation into hosts of different character. In addition to genetic differences, hosts are further modified on occasion by alteration of the hormone status, diet and radiation. Survival of transplanted tissue is demonstrated by observing the morphology, growth and the incorporation of radioactive labeled materials.

Major Findings: Study of a transplantable teratoma containing neural elements and cartilage has continued. As reported earlier cartilage elements have been observed to survive in homografts of this tumor. Recently, other more cellular elements of the tumor have been observed to occasionally survive and grow as a tumor following homografting of the tumor to C57 Black/6JN mice. Two years earlier only cartilage elements survived in homografts in C57 Black/6JN mice. The cartilage elements which survived did not grow as a tumor. The survival and growth of more cellular components of this tumor in homografts in C57 Black/6JN mice is a change which may indicate "progression" in the teratoma during its serial transplantation in isologous and isologous F_1 hybrid hosts. The appearance of the recent generations of the tumor in isologous and isologous F_1 hybrid hosts is morphologically very similar to the generations of the tumor studied two years ago. Cellular tumors in homografts, on the other hand, are distinctly different. Generally speaking these tumors in homografts are less differentiated.

Part A (continued)

In addition to simple homografts of the transplantable teratoma, tumor homografts of short duration have been retransplanted back to isologous F_1 hybrid hosts and found to have been altered by the short stay of 8 to 20 days in a homologous host. Numerous giant cells are present in the altered tumor tissue.

Transplantation of a moderately well differentiated hepatoma into rats with congenital hyperbilirubinemia has resulted in no detectable alteration of the tumor and no effect has been noted in the icterus of the host.

Significance: The relationship between a tumor or tissue transplant and the host has certain similarities to the relationship between primary neoplasms and its host. Patterns of alteration of the tissue and tumor transplant are of interest as part of a basic study of tissue response to altered environment.

Proposed Course: This project is to be continued. The alteration in the graft effected by homograft environment is of particular interest. Morphologic alterations noted in the teratoma under study make it useful for the performance of these studies.

The possibility of securing a transplantable chondrosarcoma from the Laboratory of Dr. J. Englebreth-Holm of Copenhagen is being discussed. (This tumor was shown to Dr. Swarm in Dr. Kieler's laboratory in Copenhagen when he visited there in June.) If secured, homotransplantation experiments are planned with this tumor.

Part B included: No

Serial No. NCI 513
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Ontogenetic development of plasma cells in mice

Principal Investigator: Ross C. MacCardle

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 3/8
Professional: 1/8
Other: 1/4

Project Description:

Objectives: The mesenchymal elements of connective tissues and lymph nodes, spleen, and tissues of the ileocecal region of mice from fetus to old age are being examined to determine the age at which plasma cells first appear and to determine the rate of production at later stages. The cytological characteristics of plasma cells are being studied to ascertain the state of the Golgi apparatus, mitochondria and cytoplasmic RNA at different stages of maturation of the plasma cell. An attempt is being made to determine the cellular origin of plasma cells, and to determine whether plasma cells can be formed under artificial stimulation prior to their normal date of appearance. It is not yet known whether each type of protein antibody is produced by a single separate plasma cell, or whether all are produced in the same cell at different stages of maturation which may or may not be reversible.

Methods: Freund's adjuvant will be utilized as a stimulant to formation of plasma cells. This can be injected into the fetus as well as in the newborn. Tissues will be taken at various ages and fixed and stained cytologically.

Major Findings: In the mouse, plasma cells first appear in the lymphatic tissue of the intestinal mesentery at about ten days after birth. None were found before birth and none immediately after birth. Plasma cells at ten days of age (called tentatively puberty plasma cells) can be distinguished from those of the adult mouse by the size of their Golgi apparatus which is distinctly smaller than that of the adult plasma cells.

Part A (continued)

It is not known whether such agents as Freund's adjuvant could induce the ten-day-old mouse to form adult plasma cells, or whether a puberty plasma cell merely differentiates into an adult form.

Significance: These findings and the studies planned are believed to be of fundamental importance to an understanding of the genesis of a plasma-cell tumor in the mouse, and possibly also of multiple myeloma in man.

Proposed Course: It is intended to pursue this study, particularly with a view to determining the relative susceptibility of newborn and adult mice to the formation of plasma cell tumors. This work is not yet completed.

Part B included: No

Serial No. NCI 514
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Induced and spontaneous neoplasms in laboratory animals. Factors which influence their incidence and the chemical changes they induce.

Principal Investigator: Ambadas S. Mulay

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960);
Total: 1 1/3
Professional: 1/3
Other: 1

Project Description:

Objectives: 1) To study the role of sex hormones on the incidence of liver tumors induced by some azo dyes in male and female rats.

2) Influence of diet and carcinogen on the chemistry of the adrenal gland.

3) To study enzyme systems like arginase in papillomas, spontaneous or induced by various agents, at different sites and in different species of animals.

Methods: 1) Semi-synthetic diet containing CH_3^1-paradimethylaminoazobenzene was fed to male and female Osborne-Mendel rats. Animals were intact, gonadectomized, or adrenalectomized unilaterally. Some of these rats received testosterone or stilbesterol pellets and others did not. Animals were examined for the size, the incidence, the frequency of tumors at the termination of the experiment.

2) Rats fed semi-synthetic diets, with and without carcinogen are killed at regular intervals and adrenal glands and livers analyzed for ascorbic acid and cholesterol concentration.

3) Experimental animals, mice, rats, and rabbits, are fed or painted with carcinogenic hydrocarbons like benzanthracene to induce papillomas. Tumors thus induced and other spontaneous tumors are homogenized, proteins in them go into solution. Protein, and total and active arginase is determined on the samples thus prepared.

Part A (continued)

Major Findings: 1) A report based on lower incidence of hepatoma in female rats fed azo dyes DAB, DAN and DA-2N was published last year (Proc.Soc.Exp.Biol.Med. 101: 763-766, 1959). All rats fed CH_3^1-DAB developed hepatomas after 90 days on carcinogenic diet. Most of the intact male rats developed at least one tumor nodule 2 cm. or larger in diameter while largest tumor nodule in female rats was about 1 cm. in diameter and found in only 30 percent of the rats. Gonadectomy of the male rats had no significant effect on the size, the incidence, or the number of tumors induced. Implants of stilbesterol pellets in gonadectomized males effected slight reduction in the size of the tumors still they had more and larger tumors than the females. In female rats, gonadectomy, testesterone pellets, or unilateral adrenalectomy did not change significantly either the size or the number of tumors induced.

2) Ninty days after the start of the carcinogenic diet, ascorbic acid concentration of the adrenal glands of the rats was reduced by about 30 percent and cholesterol increased by 20 percent from the respective values for adrenal glands of control rats.

3) Work on enzyme systems in papillomas has just started and there are no findings to report at this time.

Significance: 1) Sex hormones seem to account only for a part of the sex difference observed in the incidence of hepatoma in Osborne-Mendel rats fed carcinogenic azo dyes. Unilateral adrenalectomy did not change significantly the size, number, or the incidence of tumors in the female rats. This does not rule out influence of cortical hormones. However, the explanation of the residual sex difference in the incidence of hepatoma may very well lie in the enzyme systems and their relative concentrations in the liver.

2) Chemical studies on the adrenal glands of rats fed carcinogenic diets may give us clues as to relation of adrenal gland chemistry to the incidence of hepatoma.

3) Enzyme studies on papillomas should either confirm or refute the claim "that virus induces Arginase" (Nature 183: 1815, 1959).

Proposed Course: Ascorbic acid and cholesterol studies on rats receiving carcinogenic diets will continue and in the next calendar year. Enzyme studies various papillomas has just started. It will continue through the next calendar year.

Part B included: No

Serial No. NCI 515
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Adrenocortical carcinoma, its effect on the chemistry and physiology of the host rat and the steroid activity of the tumor.

Principal Investigator: Ambadas S. Mulay

Other Investigator: Edward B. Price, Jr.

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: To characterize the steroids secreted by this tumor using host rat as a biological test animal.

Methods: Tumor was transplanted in male and female rats, intact and adrenalectomized. As the tumor grew, animals were inspected at regular intervals and compared with proper control animals kept under identical conditions. Urines were collected from these rats using metabolic cages and examined for volume, specific gravity, sodium, potassium, protein nitrogen and non-protein nitrogen. Animals were killed for the final examination and body weight, tumor weight and organ weights recorded and tissues examined macroscopically and microscopically. Blood was obtained by cardiac puncture and examined for blood sugar and packed cells and serum for sodium, potassium, and protein nitrogen.

Stress was produced in intact and adrenalectomized rats, with and without tumor transplant, by intraperitoneal injection of potassium chloride solution, and animals were observed for the toxic effects. Another group of rats intact and adrenalectomized, with and without tumor transplant is under study for glucocorticoid secretion by glycogen deposition method, adapted to qualitative auto-assay of the hormones.

Major Findings: Rats bearing this adrenocortical adenocarcinoma had atrophy of the zona fasiculata and zona reticularis of the adrenal cortex, a sharp reduction in urinary sodium excretion both in intact and

Part A (continued)

adrenalectomized rats, increased potassium excretion, and hypernatremia and hypokalemia. A paper based on these findings is in press.

Stress studies on the tumor-bearing rats, demonstrated the ability of this tumor to protect the rats against KCl intoxication, a proof of mineralocorticoid secretion by this tumor. A paper based on these findings is published. Thus far, experimental evidence for glucocortocoid secretion is in the negative. There seems to be a competition for available carbohydrate between tumor metabolism and glycogen deposition systems, and glucocorticoid secretion if any is not enough to induce significant glycogen deposition.

Significance: These findings define in part the differences between the steroid secretion by this adrenocortical tumor and normal adrenal cortical tissue of rat. This opens a way for inquiry into factors responsible and the metabolic pathways involved, which bring about these differences.

Proposed Course: Study of glucocorticoid secretion will continue. Experiments to study metabolic pathways of certain steroids by tumor tissue are under consideration.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Mulay, A. S.
Corticosteroid activity of transplantable adrenocortical carcinoma in Osborne-Mendel rats.
Endocrinology 66: 769-772, 1960

2. Mulay, A. S., and Price, Edward B., Jr.
The histopathology and biochemistry of rats bearing an adrenocortical tumor.
Cancer Res. 20: In press 1960.

Serial No. NCI 516
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies on the Induction of Multiple Tumors in Certain Mammals with Stewart-Eddy Polyoma Virus-tissue Culture Preparations

Principal Investigator: Mearl F. Stanton

Other Investigators: Dr. H. Otsuka, Robert Blackwell, Sarah Stewart, Bernice Eddy (DBS), Joseph Albrecht and staff

Cooperating Units: DBS, Laboratory of Biology, NCI

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: This is a continuation of projects outlined in Individual Project Report NCI 516, 1957.

Major Findings: Summaries of major findings in cooperation with other investigators will be found in their reports. In addition, separate investigations of host factors involved in induction by this laboratory have established the following: 1) The transplantable hamster tumors referred to in NCI 516 (1958) have continued in transplant. Serological attempts to detect antibody in grafted hamsters have been made on a limited basis. Thus far significant HAI antibody has not been detected in the grafted animals. Present studies are being carried out on grafted newborn hamsters. Much of the work with grafted tumors is directed toward determining what influence host factors may have on the behavior of grafted tumors and induced tumors. Two systems are used: (a) varying host factors (age, sex, carcinogen exposure, mutogen exposure, etc.) in animals grafted with tumor lines of known behavior; (b) inducing tumors of varying type and behavior from single tissues exposed to virus-cultures and implanted into the cheek pouch of weanling hamsters.

2) An analysis of osteogenic tumors has been made following the discovery in this laboratory that mice of Strain A develop multiple bone tumors without developing the variety of tumors observed in other strains and without affecting the spontaneous incidence of pulmonary alveologenic tumors. The bone tumors are particularly amenable to studies

Part A (continued)

on progression and work in this direction will culminate in publication of a paper with B. Eddy early this year.

Significance: The polyoma virus-tumors serve as exceptional experimental tools. The wide range of species that respond and convenience in working with small rodents is an advantage over all other virus tumors. Secondly there is the advantage over chemically induced tumors both in the short latent period and opportunity of following the progress and behavior of the tumors in relation to virus as detected by new virological and morphological techniques including fluorescence antibody techniques. Much of our more recent activities center on perfecting the fluorescence technique. Finally there is good evidence to indicate that induction and progression proceed in a slow step-wise fashion which may allow separate study of the two most important attributes of tumors namely cell proliferation and the acquisition of agressive behavior patterns by the proliferating cells.

Proposed Course: The development of a simple basic system for tissue culture preparation enabling this laboratory to carry out experiments on host factors with a single standard line of virus culture preparations. Development of fluorescent antibody techniques for the detection of polyoma virus antigen. Preparation of a report on the histogenesis of tumors which develop in the hamster with Dr. Otsuka of this laboratory is in its final stages.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Stanton, M. F., Stewart, S. E., Eddy, B. E., and Blackwell, R.
The oncogenic effect of tissue culture preparations on polyoma virus on fetal mice.
J. Nat. Cancer Inst.: 23: 1441-1476, 1959.

2. Stanton, M. F.
The local oncogenic response of hamster tissues to polyoma virus.
Cancer Res. 20: 487-491, 1960

3. Stewart, S. E., Eddy, B. E., and Stanton, M. F.
Neoplasms in rodents induced by SE Polyoma virus.
ACTA Unio Internationalis Contre le Cancer XV: 842-851, 1959.

4. Stewart, S. E., Eddy, B. E., and Stanton, M. F.
Progress in virus research: The Polyoma Virus, Expe
Experimental Tumor Research Vol. I, 67-85 (Karger/Basel/New York) 1960

5. Andervont, H. B., Dawe, C. J., Dunn, T. B., Law, L. W., Potter, M., and Stanton, M. F.
Aetiology of leukemia.
Lancet 1: 154, 1959

6. Stanton, Mearl F.
Viruses and the causes of cancer.
Am. J. Med. Tech., Jan. 1961.

7. Innes, J. R. M., and Stanton, M. F.
Dacryl-sialo-adenites: A new disease entity of rats.
Am. J. Path. 1961.

Serial No. NCI 519
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Induction of transplantable hepatoma and study of some enzyme systems of the tumor and the host.

Principal Investigator: Ambadas S. Mulay

Other Investigator:

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: To develop a reproducible tumor with an enzyme system or systems which can be used as an indicator to determine the activity of this tumor.

Methods: Hepatomas are induced by feeding a diet containing carcinogenic azo dye CH_3-paradimethylaminoazobenzene to rats. Solid pieces from the tumors thus induced are implanted with a trocar at different sites. Cell suspensions are prepared from these tumors and then implanted as free cells. Enzyme studies are made on tumors and on the serum and the liver of the host rats.

Major Findings: A transplantable solid hepatoma (L-26) is established and an ascites hepatoma is in the process of being established. Enzyme studies are in the planning stage and there are no findings to report.

Significance: A transplantable tumor with reproducible characteristics will be a valuable system for the study of chemical agents.

Proposed Course: To improve and standardize the solid transplantable hepatoma and establish an ascites hepatoma. Study enzyme systems like phosphohexose isomerase, transaminase, and lactic acid dehydrogenase on these tumor-host systems.

Part B included: No

Serial No. NCI 520
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Morphologic study of the effects of drugs influencing radiation and radiomimetic compounds

Principal Investigators: Roger W. O'Gara and Margaret G. Kelly

Other Investigator:

Cooperating Units: General Medicine Branch, NCI

Man Years (calendar year 1960):
Total: 1/5
Professional: 1/10
Other: 1/10

Project Description:

Objectives: To investigate methods of modifying the effects of radiation and radiomimetic chemicals.

Methods: Usual biologic, pharmacologic and histopathologic methods; radioactive tracer techniques.

Major Findings: Previous annual reports have given data on the protective action of AET against the toxic effects of nitrogen mustard and observations on the gross distribution of labeled AET in the tissues of experimental animals. Additional studies were made of the tissues of mice following subcutaneous injections of S-35 and C-14 labeled AET. Microautoradiographs of liver, spleen, kidney, and other tissues demonstrate a localization of C-14 labeled AET in the cytoplasm of the cells and relatively little associated with the nucleus. Microscopic observations confirm the gross observations made on autoradiographs of blocks of tissue. The radioactive labeling is highest in the liver, spleen, kidney and gut, and lowest in tumor and skeletal muscle.

Significance: The study supplies information which may explain in some measure how AET protects the host animal against the toxic action of nitrogen mustard, while not protecting a transplanted tumor.

Proposed Course: (1) Tritium-labeled AET will be used to get additional information on the localization of AET within the cell.

Part A (continued)

(2) Distribution of labeled AET will be studied in animals bearing induced or spontaneous tumors.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Kelly, M. G., Rall, D. P., Trivers, G. E., O'Gara, R. W., and Zubrod, C. G.
Action of S,2-Aminoethylisothiuronium Br. HBr. (AET). Toxicity and protective effect against nitrogen mustard toxicity.
J. Pharm. and Exper. Therap. 129: 218-230, 1960.

Serial No. NCI 521
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Morphologic and biologic studies of leukemias and lymphomas of the mouse in tissue culture

Principal Investigator: Clyde J. Dawe

Other Investigators: Michael Potter

Cooperating Units:

Man Years (calendar year 1960):
Total:
Professional:
Other:

Project Description:

Objectives, Methods, and Major Findings: The objectives, methods, and major findings of this project have been given in the 1959 annual report and will not be repeated here because the project has been deferred in order to give more time to investigations on the mechanisms of action of polyoma virus.

The continuous culture cell lines derived from reticulum cell sarcoma P329 and from lymphoid tumor P388 are still being carried in our laboratory with a view toward resuming the investigations, particularly of changes in "virulence" of these cell lines in culture.

No attempts have been made in the past year to isolate cell strains from additional cell types of leukemia and lymphoma.

Part B included: No

Serial No. NCI 521a
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Tissue culture studies investigating the mechanisms of tumor induction by parotid tumor agent (polyoma virus)

Principal Investigator: Clyde J. Dawe

Other Investigators: L. W. Law, and W. P. Rowe

Cooperating Units:

Man Years (calendar year 1960):
Total: 3 1/3
Professional: 2
Other: 1 1/3

Project Description:

Objectives: This project is a continuation of Project 521a described in the 1958 and 1959 reports. It is aimed at determining the influences of certain host factors on the development of the two basic types of response to the polyoma virus in vitro; namely, the cytolytic and proliferative responses. The host factors under investigation are: chronologic age, species differences, immunologic state with respect to polyoma virus infection, stage of cellular differentiation at time of infection, and specific influence of mesenchymal elements on the development of the different types of response by epithelium.

Methods: The investigation employs the gelatin sponge matrix method of tissue culture described in publication (2) of the 1959 report, and modifications of the salivary gland rudiment culture techniques described by Bovghese and by Grobstein. The use of plasma clot in conjunction with the sponge matrix method has been discontinued, as the presence of plasma clot has been found to have no special influence on results. Cultures of rudiments or of developed salivary gland tissues are infected with polyoma virus and compared with similar cultures not infected with the virus.

Epithelial and mesenchymal components of salivary gland rudiments are separated from each other by a trypsinization technique, and the effects of virus on each isolated component are studied in comparison with the effects of the virus on the intact, whole rudiments.

Part A (continued)

In experiments to clarify the modus-operandi of species differences on response to the virus, mesenchyme from rat salivary gland rudiments is combined with epithelium from mouse salivary gland rudiments, and the reciprocal. The effect of virus on each isolated component of each species is also studied, along with uninfected control cultures of each component as well as of the intact rudiments from each of the two species. C3H/Bi mice or AKR x C3H/Bi F_1 hybrids are used as the source of mouse salivary gland rudiments, and Fischer rats are used to supply rat salivary tissue or rudiments.

In the studies of effect of polyoma virus antibody on polyoma virus-infected cultures of adult mouse salivary gland, anti-serum is obtained from serum of mice bearing polyoma virus-induced primary tumors. The anti-serum is applied to cultures at various time intervals after infection of the cultures with virus, and the results are compared with controls consisting of uninfected cultures similarly treated with antibody as well with controls consisting of infected cultures similarly treated with normal mouse serum.

Major Findings: The results of studies on effect of chronologic age and on stage of differentiation have been reported in publications (1) and (4). It was found that salivary gland tissue from adult mice showed an epithelial proliferative response that was even more pronounced than that observed with the same tissue from newborn mice. Intranuclear inclusion bodies and other nuclear and cytoplasmic changes occurred in the adult tissues as well as in the newborn tissues. On the other hand, at the 30-day interval after infection, cultured salivary gland rudiments from 13- and 14-day old mouse embryos showed a less pronounced epithelial proliferative response than that seen in cultures of salivary gland from either newborns or adults. Infected cultures of embryonic rudiments studied after longer periods in culture (43 days) showed a proliferative response more nearly comparable to that seen in 30-day cultures of adult tissue. Intranuclear inclusions and other cytologic changes were similar in the rudiment cultures to those seen in cultures of older tissues. Necrosis of mesenchymal cells was observed in glands of all ages, but a proliferative response by mesenchymal cells could not be detected at any age or stage of development.

Experiments with isolated epithelial and mesenchymal components of submandibular salivary gland rudiments are still incomplete. Several trials with both the gelatin sponge technique and the plasma clot technique have so far showed uniform results, however. The infected epithelial components have failed to show any proliferative response at all. In fact, survival of the epithelial component is limited to a time ranging from a few days to a few weeks, and this time has not been influenced by the presence or absence of polyoma virus. Thus far there is no positive evidence that the isolated epithelial cells are actually infected by the virus, but this impression at present is based only on observations of living cultures, and needs confirmation by examination of fixed and stained cultures.

Part A (continued)

On the other hand, cultures of isolated mesenchymal components of mouse salivary gland rudiments show a cytolytic response to the virus, but not a proliferative response. As the observations stand at present, it appears that the proliferative response and perhaps also the susceptibility infection of salivary gland epithelium may be dependent on its maintenance of contact with its proper mesenchyme.

To further probe the influence of mesenchyme on the susceptibility and response of the rudimentary epithelium to polyoma virus, combinations of rat mesenchyme with mouse epithelium are prepared and infected with the virus. Previous experiments with intact rudiments (unpublished) and with salivary tissue of suckling rats (publication (4)) have demonstrated that rat salivary epithelium does not respond to polyoma virus in this culture system, either by proliferation or cytolysis. Since morphogenetic induction of salivary gland is not species specific (e.g. chick mesenchyme will induce morphogenesis of mouse submandibular epithelium (Sherman and Auerbach) the possible influence of virus-susceptible mesenchyme on (mouse) virus-non-susceptible epithelium (rat) can be tested in reciprocal exchange experiments using the mesenchymal and epithelial components of rat and mouse. It is conceivable, for example, that under the influence of mouse mesenchyme, rat epithelium may become capable of infection by and/or response to the virus. Or, in the reciprocal situation, rat mesenchyme might render mouse epithelium incapable of infection and/or response to this virus. Technical difficulties are great in this approach because of the necessity to obtain simultaneously, rat and mouse embryos at the proper stages of development. A practicable technique has been worked out and it has been observed that mouse mesenchyme will support rat rudimentary epithelium in vesicular and tubular forms for at least two weeks after infection with virus. Microscopic results are not yet available.

As still another approach to the question of epithelial-mesenchymal relationships in the response to polyoma virus, time-lapse cinematographic studies of the effect of the virus on mouse salivary gland rudiments are being undertaken. This work has been greatly aided by the development, by Mr. Willie D. Morgan, of a gas exchange system used in conjunction with the Rose culture chamber. This system permits continuous long-term observation and photography of the rudiments under conditions simulating those achieved in tube cultures (publication 2). Many technical difficulties have been met and overcome with the invaluable assistance of Mr. Morgan, and it is expected that significant observations will be made soon in the course of this work.

Studies on species differences in response to polyoma virus in culture have recently been reported (publication 4). It was found that salivary glands from rats, hamsters, Mastomys, and human fetuses failed to undergo any of the changes previously observed and reported in mouse salivary glands in similar cultures. Taken in conjunction with the studies on antibody effect described below, then results indicate that refractoriness

Part A (continued)

of the salivary tissue of the animals tested depends primarily on characteristics inherent to the tissues concerned, and not on the effectiveness of the animal's immunologic response.

Preliminary results of a study on the effect of specific antibody on the response of mouse salivary gland to polyoma virus in vitro were presented at the 10th International Congress for Cell Biology and will be published shortly (publication 4). Mouse antiserum was introduced into cultures of mouse salivary gland at intervals of 5, 10, and 22 days after the cultures had been infected with polyoma virus. The antiserum was then added to these cultures continuously until a total period of 50 days had elapsed. Cultures were then serially sectioned and examined microscopically. It was found that cultures treated with antiserum from the 5th day onward showed a marked diminution of the proliferative response, although cytologic evidence of virus infection persisted. Cultures treated with antiserum from the 10th day onward showed only slight diminution of the proliferative response, while those treated from the 22nd day onward showed no differences from the infected but untreated controls. These results appear to be in accord with observations in vivo which show that newborn mice passively immunized by infected mothers fail to develop tumors after inoculation with the virus, and that tumor growth continues in mice bearing them, even though the titer of circulating antibody in these mice is very high. A question arises as to why the cultures treated early failed to develop a proliferative response even though cell infection had clearly been established, and continued through the culture period. It appears probable that a quantitative dose-response effect is involved, based on interruption, by the antiserum of continued cycles of virus production and infection of neighboring cells.

Since last year's report we have succeeded in collaboration with Dr. L. W. Law, in obtaining tumor growth at the site of transfer of infected salivary gland cultures in 3 instances. Gelatin sponge cultures were transferred subcutaneously into newborn mice. In one of the three resulting tumors the histologic pattern was that of a polyoma virus-induced salivary gland tumor. The other two tumors showed the histologic characteristics of fibrosarcomas. The salivary gland-type tumor and one of the fibrosarcomas have been serially transplanted by Dr. Law, and it has been found that the former continues to contain polyoma virus whereas the latter does not.

The fact remains that the great majority of polyoma virus-infected organ cultures of mouse salivary gland do not grow into tumors after transplantation. A possible technical reason for this was recently noted. Microscopic examination of the gelatin sponge cultures a few days after transfer showed that the sponges were compressed in the subcutaneous tissues and the cells within them were already dead. Apparently the cells are unable to obtain nutrition when tightly sandwiched between several layers of gelatin. Renewed attempts at transplantation are planned in which the cells will be freed from the meshes of the sponges before transfer.

Part A (continued)

Efforts are also underway to establish continuous culture cell lines from infected and control salivary gland organ cultures. Such cell lines would allow us to study the differences between (a) cells which acquire neoplastic properties in vitro without the influence of polyoma virus.

Significance: The experiments dealing with influence of age and stage of development of mouse salivary gland on its responsiveness to polyoma virus suggested that, other variables excluded, one would expect tumors to develop as readily in adult glands as in those of newborn mice. The difficulty in demonstrating this in vivo lies in the requirement of completely eliminating the variable to immune response in intact adult mice. However, in our work in collaboration with Dr. L. W. Law (publication 3), it has been shown that it is possible to induce salivary gland tumors in nearly 50% of adult mice following total body x-irradiation of the recipients. The tumors had the same histologic appearance and the same anatomic locations as those induced in non-irradiated mice inoculated at birth, indicating that x-ray did not contribute to tumor development by direct action. Furthermore, no tumors of similar type occurred in mice treated with x-ray alone. Taking this evidence in conjunction with the observations in cultures treated with antibody, and the knowledge that antibody response is delayed in newborn mice, the conclusion is reached that the high susceptibility of newborn mice to polyoma virus tumor induction depends primarily on the weakness of the immune response at this age, and not on the presence of "primitive" cells, or on a higher susceptibility of rapidly growing cells.

The strongest point of interest in this area lies in the question as to just how differentiation affects susceptibility to viral and other tumor agents. The very fact of special susceptibility of certain organs and cells to specific viral and other tumor agents forces one to look to the processes that determine differentiation for the causal factors in susceptibility and response. Many advances in the study of developmental processes have been made in the field of experimental embryology, and these can be exploited in research on tumor genesis.

Proposed Course: The projects listed in the 1959 report are still being pursued, and, as indicated in the present report, some have yielded preliminary results. It is our plan to complete these investigations, for the most part, before outlining additional projects with the polyoma virus.

It seems, however, desirable to take exploratory steps at this time in search of clues as to whether and how developmental processes affect susceptibility and response to tumor agents of types other than viruses,--for example, the chemical carcinogens. Evidence in this line should strengthen or weaken support of the hypothesis that all tumor agents act through some final common channel. Investigations on the urethane-pulmonary tumor system in mice are especially attractive, as it is possible to separate the epithelial and mesenchymal components of

Part A (continued)

rudimentary lungs. Studies are also needed here to determine whether the stages of morphogenesis and differentiation correlate with susceptibility to the action of urethane. Furthermore, it would be valuable to determine the effects of urethane on organ culture of lung. If effects *in vitro* could be closely correlated with those *in vivo*, the organ culture system would serve as a model test system for chemical carcinogens. Such test systems might be useful in assaying carcinogenic activity of chemicals on human tissues.

Some organ culture studies of the effect of urethane on embryonic and newborn mouse lungs are currently in progress in our laboratory. They will be continued and extended according to the results obtained and the time available.

Part B included: Yes

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Dawe, C. J.
Cell sensitivity and specificity of response to polyoma virus.
Symposia Tumor Viruses, Nat. Cancer Inst. Monograph No.4: 67-128, 1960

2. Morgan, W. D., and Dawe, C. J.
An intermittent perfusion and gas exchange method for continuous cultivation of cells in the Rose chamber.
J. Nat. Cancer Inst. In press.

3. Law, L. W., and Dawe, C. J.
Influence of total body x-irradiation on tumor induction by by parotid tumor agent in adult mice.
Proc. Soc. Exp. Biol. and Med. In press.

4. Dawe, C. J., Law, L. W., and Rowe, W. P.
Influence of age, species, and immune factors on the response of salivary gland to polyoma virus in tissue culture.
Pathologie et Biologie. In press
(Presented at 10th International Congress for Cell Biology, Paris, France, September 9, 1960)

Serial No. NCI 522
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Studies on the induction and natural history of conjunctival carcinoma in Hereford cattle

Principal Investigator: Mearl F. Stanton

Other Investigators: Robert Zeigel, Robert Manaker, Ray Bryan, W. I. Gay, and Francis Abinanti

Cooperating Units: Laboratory Aids Branch, Division of Research Services, Laboratory of Biology, NCI

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To determine the fate of certain proliferative epithelial lesions on the eyes of cattle and to attempt by artifical means to induce carcinoma in cattle with the products of the tumors which occur naturally.

Methods: Viable tumor tissue and cell free extracts of conjunctival carcinomas of Hereford cattle, prepared by concentration techniques successfully established for Rous sarcoma virus, have been inoculated into the conjunctivas and udders of Hereford cattle. Both young unaffected animals and animals with conjunctival carcinoma have been used as recipients of the inocula. Dr. R. Zeigel does electron microscopy on biopsied tumors.

Major Findings: A herd of 500 cattle in Eastern Maryland has been studied. Ninty percent of the herd has eye tumors. Systematic follow up of progressing lesions with photographs is in progress. Biopsied material has been used for electron microscopy and tissue culture study as well as inoculation. The conjunctiva of 4 cattle have been inoculated with tumor extracts or culture material. Thus far we have uncovered no evidence other than epidemiological evidence that this tumor is etiologically related to micro-organisms. However, further work in in progress through morphological studies by Dr. Zeigel and tissue culture studies by Dr. Manaker and Dr. Abinanti.

Part A (continued)

Significance: This is an unquestionably malignant epithelial tumor which has its counterpart in man, and follows the natural history of human epidermoid carcinoma. The high incidence of such a lesion in a herd of cattle suggests a common etiological factor not as yet apparent. A most likely possibility is that the initial verrucous lesion is induced by insect-borne viruses. Subsequent progression to malignancy may be a part of or separate from the initiating process. The cow serves as an excellent animal in which to study this problem, of added significance is the possibility of uncovering means of preventing the disease in cattle, a serious economic hazard in present day animal husbandry.

Proposed Course: It seems likely that the precursor to the malignant lesion is the hyperkeratotic verrucous wart noted in many of the Virginia cattle. We shall attempt to collect, recover, and induce typical lesions with such material as well as with concentrated products of the malignant lesion.

Part B included: No

Serial No. NCI 523
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Effects of ionizing radiation; morphologic alterations and neoplasms

Principal Investigator: Richard L. Swarm

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 1 1/3
Professional: 1/3
Other: 1

Project Description:

Objectives: The collection of information regarding the changes effected by ionizing radiation of different types in man and experimental animals.

Methods: (1) Detection and study of neoplasms and other morphologic changes in experimental animals who have received external radiation or internally administered radioactive isotopes.

(2) A study of the distribution, metabolism and effects of radioactive isotopes of several types in man and animals. Here the emphasis is on the utilization of gross and histologic autoradiographic techniques in the study of thorium dioxide, S^{35} and I^{131} labeled materials.

(3) Participation in the activities of the Pathologic Anatomy Department. Special emphasis is made in the study of human specimens demonstrating change due to irradiation or containing radioactive isotopes.

(4) To facilitate the above and to assist other investigators, collaboration on problems in this field is fostered. Studies on the distribution of S^{35} in human tissues have been in collaboration with clinical studies of Dr. J. Robert Andrews and others in the Radiation Branch, NCI.

Part A (continued)

Major Findings: As a part of the continuing interest in the effects of colloidal thorium dioxide in man, visited in several European laboratories for brief periods to study tissue specimens of human patients who were given thorium dioxide and developed neoplasms. These laboratories included the following: University of Lisbon, Portugal - Prof. Horta; University of Zurich, Switzerland - Prof. Rüttner; Finsen Institute, Copenhagen, Denmark - Dr. C. Johannsen and others.

A report of the pattern of reaction to subcutaneously extravasated colloidal thorium dioxide in man and animals was given at the International Academy of Pathology meeting in London.

Study of rabbits given intravenously injected thorium dioxide has continued. The one animal reported alive in the annual report of last year is now dead with a hemangioendothelial tumor of the spleen and liver. This animal received a single injection of thorium dioxide and the latent period was a year longer than animals which received two injections of thorium dioxide.

Injection of thorotrast into newborn rabbits has not resulted in a marked reduction of the latent period for vascular tumors.

Work with transplantable tumors produced in mice by intravenous injection of thorium dioxide has continued. Morphologic study of the transplantable hemangioendotheliomas has proved particularly interesting. One such tumor has been observed to metastasize occasionally to the spleen and lungs following subcutaneous transplantation. Working with this same tumor, Dr. P. Mori-Chavez has found visceral metastases following subcutaneous transplantation more frequent at high altitudes than at sea level.

Dr. Swarm participated in a conference dealing with the effects of thorium in man and animals at the University of Rochester in January 1960.

In collaboration with Dr. Andrews, tissues from three additional patients with chondrosarcoma given S^{35} have been studied in detail.

A few rats given large amount of S^{35} have been observed for neoplasia. In the first 18 months, none of these two animals has developed neoplasms of cartilage, reticular tissues or skin.

Part A (continued)

Significance: With increased exposure of man to radiation, the study of the pathogenesis of irradiation injury and irradiation induced neoplasia has become urgent.

Proposed Course: (1) Activities under methods will be continued.

(2) Study of neoplasia following thorotrast in experimental animals will be continued.

(3) If the transplantable chondrosarcoma referred to in NCI Report 512 becomes available, the effects of large doses of S^{35} on this tumor will be tested.

Part B included: No

Serial No. NCI 526a
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Chemical carcinogenesis

Principal Investigators: Roger W. O'Gara and Margaret G. Kelly

Other Investigators:

Cooperating Units: General Medicine Branch, NCI

Man Years (calendar year 1960):
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: To determine the effect of chemical carcinogens in newborn animals.

Methods: Usual pharmacologic, biologic, and histopathologic techniques.

Major Findings: (1) For the effects of 3-methylcholanthrene and dibenz(a,h)anthracene on newborn mice see report of Kelly, General Medicine Branch, NCI.

(2) Newborn hamsters were given a single, subcutaneous injection of either 0.1 mg. or 0.45 mg. 3-methylcholanthrene in 0.05 ml. of olive oil. By 16 to 24 weeks, about 75% of the animals given the lower dose and 85% of those which received the higher dose had developed large (or multiple) subcutaneous fibrosarcomas. The earliest tumors appeared in about 12 weeks. The majority of the animals given the higher dose had marked cystic dilation of the kidney tubules, but there was no evidence of neoplastic changes at the junction of cortex and medulla. About 40% of the animals given the higher dose were stunted, attaining a body weight of less than 50% of normal.

(3) 2-fluorenylacetamide has been given to newborn albino mice by subcutaneous, intraperitoneal, and oral routes respectively, on day of birth, and on 3rd, and 6th day after birth. The total dose varied from 1.5 mg. (I.P.) to 4.0 mg. (oral and S.C.). The compound was given as a suspension in aerosol-OT-methocel solution. The results after six months were inconclusive, due apparently to the toxicity of the vehicle used.

Part A (continued)

(4) 3-amino-1,2,4-triazole was given continuously to albino mice for 24 weeks beginning at birth. No mortality, loss of body weight, or abnormality of the thyroid gland were obtained after a total dose of 1,024 mg. (oral and subcutaneous routes) or 536 mg. per animal by the intraperitoneal route. Except for local inflammatory reaction in mice administered the compound by subcutaneous route, and some degeneration of the adrenal medulla after oral administration, no significant abnormalities were observed.

(5) 3-amino-1,2,4-triazole has been administered to rats, starting with newborn animals. A total of 230-645 mg. was given over a period of 40 days (oral and subcutaneous routes). During treatment, the rats fail to show normal weight gain, and animals killed within 48 hours after cessation of treatment had enlarged hyperplastic thyroid glands. The remainder of the rats began to gain weight within one week after treatment was stopped and have been apparently normal since then,-- approximately six months. These animals will be sacrificed in the near future and histopathologic examination of the thyroid gland will be made.

(6) Collaborative studies with Dr. Hesselbach of NIAID are continuing. Fibrosarcomas were induced by subcutaneous injection of light green and fast green dyes in rats. The original tumors and several lines of the transplanted tumors have been studied for changes in oxidative enzyme systems. An attempt has been made to correlate changes in the morphology of successive transplant generations with corresponding changes in the levels of enzymatic activity.

Significance: (1) It is concluded from these experiments that mice are not susceptible to the goiterogenic action of 3-amino-1,2,4-triazole.

(2) Newborn hamsters are quite susceptible to the local induction of fibrosarcomas by subcutaneous administration of 3-methylcholanthrene. Another route of administration must be used if one is to study the induction of other tumors in the hamster.

(3) Recognition of age difference in susceptibility to chemical carcinogens may be of importance: (a) in selecting "safe limits" for exposure of human populations to environmental carcinogens; (b) in providing a more sensitive and efficient assay system for carcinogens of low potency; (c) in understanding factors /local and systemic/ responsible for tumor induction, and (d) in providing an easily reproducible system for the screening of chemotherapeutic agents on induced tumors.

Proposed Course: (1) Newborn rats will be given injections (subcutaneous, intraperitoneal or oral route) of 2-fluorenylacetamide and N-Nitrosodimethylamine and observed for development of tumors.

Part A (continued)

(2) A collaborative study has been initiated with Dr. Walter Newton, NIAID (Lab. Germ-free Animals Research) to measure the carcinogenic effect of a single subcutaneous injection of 3-methylcholanthrene in newborn germ-free mice. These mice are reported to be free of known viral agents.

(3) In collaboration with Mr. Mantel (Biometrics Branch, NCI) a study has been initiated to determine the lowest dose of dibenz(a,h) anthracene and 3-methylcholanthrene that will induce tumors when injected subcutaneously into newborn mice. The observation period will be 48 weeks (now in the 25th week). At present, subcutaneous fibrosarcomas have been obtained at 0.0002 mg. DBA and 0.0004 mg. MCA. Lung tumors have been obtained with 0.0067 mg. DBA and 0.0037 mg. MCA.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publication

Publications other than abstracts from this project:

1. Kelly, M. G., and O'Gara, R. W.
Induction of tumors in newborn mice with dibenz(a,h)anthracene and 3-methylcholanthrene.
J. Nat. Cancer. Inst. In press.

2. Hesselbach, M. L., and O'Gara, R. W.
Fast green and light green induced tumors: Induction, morphology, and effect on host.
J. Nat. Cancer Inst. 24: 769-793, 1960

Serial No. NCI 527
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: A study of the physiopathologic changes in tumor-bearing host and tumor lesion

Principal Investigator: Richard A. Malmgren

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: 1. To study further the properties of the liver mitotic stimulant in tumors. 2. To apply the fluorescent antibody technique to the study of the tumor virus, to autoimmune phenomenon, and the detection of DNA. 3. To evaluate host and cell factors in the metastasis of various mouse tumors.

Methods: 1 (a). Mouse tumors and normal tissues were injected into mice. In some instances these preparations were heated to 80°C. for 20 minutes and the supernatant fraction used for the injections. The effect of this material on the liver mitotic activity of the host was observed.

(b) Serum from humans with and without cancer was injected into rats and mice and at 24, 48, 72, and 96 hours the livers of these animals were examined for liver mitotic activity. In some cases hepatectomized animals were used. Animals treated in the same fashion were also given tritium labeled thymadine and the DNA was isolated from their liver at 72 hours. The quantity of tritium per milligram DNA was determined. (In collaboration with Dr. Edward McLaughlin, Surgery Branch, NCI).

2 (a). Antisera prepared in rabbits against P388 tissue culture cells infected with the polyoma virus was tested by means of the fluorescent antibody technique on P388 tissue culture cells infected with the polyoma virus for antiviral antibody in an attempt to demonstrate the intracellular location of the virus antigen. Sections of salivary gland tumors were also examined after exposure to the same fluorescein labeled antisera.

Part A (continued)

(b) Chicken and turkey anti-Rous virus sera was labeled with fluorescein isothiocyanate and tested on sections of Rous sarcoma in an effort to demonstrate the intracellular location of the viral antigen.

(c) Fractions of the serum from patients with Lupus Erythematosis were tested for the component responsible for the anti nuclear activity of this material. The indirect fluorescent antibody technique was the method used for evaluating these fractions. (In collaboration with Dr. Howard Goodman, NIAID.)

3. A number of spontaneous and induced mouse tumors have been injected intravenously into mice. The nuclear DNA content of the primary tumor and its metastasis have been studied using the microspectrophotometric technique. (In collaboration with Dr. Elizabeth Chu, Pathologic Anatomy Branch, NCI).

4. Three months were spent in Professor Torbjorn Caspersson's laboratory in Stockholm, Sweden, studying the histochemical properties of carcinoma in situ and invasive carcinoma of the uterine cervix by microspectrophotometric, interference microscopy and planimetry techniques.

Major Findings: 1 (a). The liver mitotic stimulant was found to be present in a variety of mouse tumors. It was heat stable and could be stored in vacuo, was not strain specific, and had a high nucleic acid content. The liver mitotic stimulant was present in heated normal tissue. Unheated normal tissue seemed to contain an inhibitor for the liver mitotic stimulant as it could block the mitotic stimulating effect of tumor tissue.

(b). An increase in the mitotic activity of the liver of mice and rats treated with serum from cancer patients was noted. This effect was not evident when the animals were treated with serum from normal individuals. There was also an increase in the new DNA in the liver of hepatectomized rats treated with serum from cancer patients as compared with that of rats treated with normal human serum.

2 (a). In a collaborative study with Dr. Alan Rabson and Dr. Giancarlo Rabotti of the Laboratory of Pathology, NCI, the intranuclear location of the polyoma virus in P388 tissue culture cells was demonstrated by the fluorescent antibody technique. The viral antigen could not be seen in sections of polyoma induced salivary gland tumors, however.

(b) In collaboration with Dr. Mary Fink of the Laboratory of Biology, NCI, the intranuclear and intracytoplasmic location of Rous sarcoma virus antigen was demonstrated in sections of Rous sarcoma tumors using the fluorescent antibody technique.

Part A (continued)

(c) In collaboration with Dr. Howard Goodman, NIAID, and Dr. John Fahey, NCI, the antinuclear fraction of the serum of patients with Lupus Erythematosis was demonstrated in the 7 S and 18 S globulin fractions.

3. There is a tendency for the metastatic tumor cells to have a higher ploidy than the primary tumor from which they are derived. However, this difference is not of such an order of magnitude as to suggest that the high ploidy cells are the only ones capable of establishing themselves in a metastatic focus.

4. In the limited study using Professor Caspersson's equipment, no significant difference was demonstrated between the various cells studied from carcinoma in situ and invasive cancer patients. Time did not permit a comparison with normal cell components of a comparable histologic type.

Significance: 1 (a) The studies on the liver mitotic stimulant in tumor tissue suggest that the tumor is elaborating a growth stimulating substance which is also present in normal tissues. The normal tissues, however, have, in addition, an inhibitor for this stimulating factor. This concept has important implications to the problem of carcinogenesis and the autonomy of cancer.

(b) The observation of the presence in the serum of human cancer patients of a factor which stimulates the DNA metabolism and mitotic activity of mouse and rat liver tissue provides the opportunity to study in the human growth factors associated with cancer. Its potential value as a diagnostic test has also been considered.

2 (a) The intracellular localization of the tumor viral antigens is of basic importance to the study and understanding of viral neoplasms. The fluorescent antibody technique provides a useful tool for obtaining this type of information.

(b) Study of the antinuclear serum factors in Lupus Erythematosis may contribute to an understanding of the collagen diseases.

3. A study of the ploidy of primary tumors and metastasis helps to classify one of the factors that has been considered of potential importance in the metastatic process. That is, the idea that the high ploidy cells may be either better able to leave the primary or better able to establish a metastatic focus once they are free in the blood stream.

Proposed Course: 1) A study of the growth factors associated with the tumor lesion will be continued with emphasis on improved techniques for detection and characterization. 2) The use of the fluorescent antibody technique for the study of virus tumors will be extended. 3) Further studies of the host cell relationship in the metastatic process will be undertaken.

Part B Included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Malmgren, R. A., Rabott, G., and Rabson, A. S.
Intracellular localization of polyoma virus antigen demonstrated with fluorescein labeled antiserums.
J. Nat. Cancer Inst. 24: 581-587, 1960.

2. Malmgren, R. A., Fink, M A., and Mills, W.
Demonstration of the intracellular localization of Rous sarcoma virus antigen by fluorescein-labeled antiserums.
J. Nat. Cancer Inst. 24: 995-1001, 1960.

3. Goodman, H. C., Fahey, J. L., and Malmgren, R. A.
Serum factors in Lupus Erythematosis and other diseases reacting with cell nuclei and nucleoprotein extracts: Electrophoretic, ultracentrifugal and chromatographic studies.
J. Clin, Invest. 39: 1595, 1960.

4. Malmgren, R. A., and Mills, W.
Studies of the properties of the liver mitotic stimulant (LMS) present in mouse tumor tissue.
J. Nat. Cancer Inst. In press.

Serial No. NCI 533
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Developmental pathology and function of the thymus and thymic tumors

Principal Investigator: Roger W. O'Gara

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To obtain information on (a) the function of the thymus, (b) the factors involved in the growth of the thymus and in the development of epithelial thymomas, (c) the influence of the thymus on the induction of lymphoid tumors.

Methods: Surgical procedures and the usual biologic, histopathologic and radiation techniques.

Major Findings: (a) The data reported in the last annual report have been prepared for publication in the near future, and will not be repeated here. Transplantation of thymus into the liver of thymectomized irradiated mice has, like transplantation into the spleen, failed to produce any increase in radiation-induced leukemia.

(b) Since non-irradiated thymic tissue transplanted into the spleen would not produce leukemia in irradiated mice, it seemed of interest to determine the fate of irradiated thymic tissue transplants. Thymectomized C57Bl mice were given an intrasplenic transplant of thymic tissue before radiation. After an interval of 17 days to allow the transplant to become established, the mice were given a total of 525 r whole body radiation. A second group of mice were not thymectomized but were given 525 r of whole body radiation. Seven days later they were given an intrasplenic transplant of thymic tissue to determine whether non-irradiated thymic tissue would protect the animals from radiation-induced leukemia. No leukemias were obtained in 14 months in either group under these

Part A (continued)

conditions, but the results are inconclusive since the irradiated intact control mice also failed to develop leukemia. No reason is apparent for the failure of the controls to develop leukemia, since previous experience has indicated that about 60% leukemia can be expected under these conditions. The experiment is being repeated.

(c) Since a virus has recently been implicated as a factor in radiation-induced leukemia (Lieberman and Kaplan 1959), it seemed of interest to determine whether a chemotheropeutic agent known to be effective against mouse leukemia would alter the course of radiation-induced leukemia. Three groups of C57Bl mice were given multiple exposures of whole body radiation (total 525 r). One group received endoxan incorporated in the diet for the duration of radiation treatment (15 days). A second group received endoxan in the diet for a six-month period after radiation. The third (control) group received no endoxan.

After a 12-month observation period, the incidence of leukemia is approximately the same in the three groups. The group receiving endoxan for 6 months after radiation developed marked greying of hair. Other evidences of chronic toxicity in this group have not yet been evaluated. This experiment should be repeated using a higher dose of endoxan, and a shorter period for the post-radiation treatment, since it is likely that an optimal dose level of endoxan was not obtained.

(d) C57Bl mice have been given a single dose of 400 r of whole body radiation either as newborns or at 2, 4, or 8 weeks of age. The effect of age on the susceptibility of mice to radiation-induced leukemia will be investigated. At present, the mortality has been less than 10% in all groups except the 4-week-old groups, but no other results are available at this time.

Significance: These experiments will supply information on the reaction of the thymus to irradiation, and on the relationship between the thymus and radiation-induced leukemia. Information gained will be useful in understanding the mechanism by which x-rays induce leukemia.

Proposed Course: (1) The experiment described above in section (a) is being repeated, and it is expected that a definitive answer will be obtained to the problem under investigation.

(2) Irradiated mice will be treated during and immediately after radiation with chemotherapeutic or other agents in an effort to influence the developing lymphocytic leukemia.

(3) Information obtained from irradiation of mice of various ages (0,2,4, and 8 weeks old) will afford us information on the optional age for the induction of leukemia by radiation. If the age at which leukemia appears is relatively constant, this method may afford another system for study of the treatment of induced leukemia.

Serial No. NCI 535
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Experimental studies on the effect of nutritional deficiencies on the pathogenesis of hepatomas in rats

Principal Investigator: Herschel Sidransky

Other Investigators:
1. Tsuneo Baba, Laboratory of Pathology, NCI, Section A(1)
2. Miloslav Rechcigal, Jr., Laboratory of Biochemistry, NCI, Section B
3. Emmanuel Farber, Dept. of Pathology and Biochemistry, Tulane University, New Orleans, Louisiana (Section C(1)

Cooperating Units:

Man Years (calendar year 1960):
Total: 2 1/3
Professional: 1
Other: 1 1/3

Project Description:

Objectives: To study the pathologic changes that occur in certain nutritional deficiencies which may be related to the subsequent development of neoplasia in the liver of the rat.

Methods: A. Kwashiorkor, a widespread protein deficiency disease in infancy and childhood, has been considered possibly to be related to the high incidence of cirrhosis and hepatoma of adults in Africa. Experimentally we have previously found that young rats force-fed synthetic diets devoid of certain single essential amino acids, threonine, methionine, or histidine, develop many lesions which are similar to those found in kwashiorkor. In order to learn additional information under our experimental conditions, deficiencies in valine or lysine have recently been studied. Also experiments designed to study the influence of the level of carbohydrate intake on the change induced by single amino acid deficiencies have been studied. In order to simulate more closely the natural diets in areas where kwashiorkor exists, experiments have been performed in which young rats have been force-fed diets containing certain poor quality plant proteins as the sole source of protein.

Part A (continued)

B. Influenced by our findings in rats force-fed synthetic diets deficient in single essential amino acids, we have become interested in certain morphologic and biochemical changes induced by feeding protein-free diets. Studies are in progress to determine the effect of the quantity of protein-free diet consumed under force-feeding conditions upon certain organs, liver, pancreas, and muscle, in the rat.

C. Choline deficiency in acute experiments with rats induces a fatty liver while in chronic experiments cirrhosis and hepatoma develop. The possible relationship between the development of an acute fatty liver and the subsequent development of hepatoma is not clear. In acute experiments the susceptibility to develop fatty livers has been found to vary in certain species of animals. A study has been conducted to learn whether the level of the liver enzyme, choline oxidase, in 8 species and in man, may be related to this altered susceptibility.

In addition, a study has been undertaken in order to obtain a more clear understanding of the relationship between methionine and choline in acute choline deficiency.

Previously it has been reported that in chronic experiments rats develop hepatomas when they are placed on a choline deficient, low methionine diet. More recent studies reveal that ethionine, an ethyl analog of methionine, induces hepatomas in the rat. Ethionine is believed to act as a competitive inhibitor of methionine and thereby induces a relative methionine deficiency state. Methionine but not choline completely inhibits this action of ethionine. To learn more about the mechanism by which the interference with methionine metabolism can induce hepatomas in rats, chronic experiments are in progress in which rats are on a methionine deficient, but adequate choline diet.

Major Findings: A (1) One publication describes the chemical pathology of acute valine and lysine deficiencies. In this study young rats force-fed a synthetic diet devoid of valine or of lysine developed pathologic changes which resembled those described to be characteristic of kwashiorkor in the liver, pancreas, salivary glands, thymus and spleen within 6 days. Animals fed the same deficient diet ad libitum consumed less food and showed no specific pathologic changes. The differences in the results between the force-feeding and the ad libitum feeding regimens were explained in terms of difference in the quantity of the deficient diets consumed.

(2) In a paper presented at the Fifth International Congress on Nutrition, September 3, 1960, we reported on a study on the influence of carbohydrate intake on the morphologic and biochemical changes in young rats fed synthetic diets devoid of threonine or valine. Rats force-fed for 3 days developed pathologic changes resembling those

Part A (continued)

described in kwashiorkor only if the diets devoid of threonine or valine contained adequate or high carbohydrate. Animals force-fed the identical deficient diets but kept low in carbohydrate did not develop pathologic changes. The results indicate that the pathologic changes induced in the experimental animals may be attributed to a nutritional imbalance created by an essential amino acid deficiency and an adequate or high carbohydrate or caloric intake.

(3) One publication describes the chemical pathology of nutritional deficiency induced by certain plant proteins. Young rats force-fed certain plant foodstuffs containing poor quality proteins and supplemented with other essential nutrients developed certain pathologic changes resembling those found in kwashiorkor within 3 days. These changes were not marked with corn, rice, and cassava diets and less marked with wheat and milo diets. Animals fed the same diets ad libitum for 7 days, in contrast with those force-fed, consumed less food and showed fewer pathologic changes.

B. Experiments are in progress in which rats are force-fed varying amounts of a protein-free diet for 3 days. Preliminary results indicate that the quantity of protein-free diet ingested influences certain pathologic and biochemical changes in the liver and pancreas. Alterations in lipid, glycogen, protein, and catalase activity of the liver and amylase and proteolytic enzyme activity of the pancreas are being followed.

C (1) One publications describes the various levels of choline oxidase in the livers of rats, mice, chickens, dogs, hamsters, rabbits, guinea pigs, monkeys, and human beings. Marked species differences were observed. In animals the rat has the highest activity while the guinea pig has the lowest activity. Choline oxidase activity in livers from human biopsy and autopsy material was lower than in any species studied. A possible correlation between the level of hepatic choline oxidase in each species and the ease of induction of choline-deficient fatty liver was considered.

(2) Experimental studies in which young rats were fed ad libitum synthetic diets devoid of choline with varying levels of methionine reveal that growth and morphologic changes due to choline deficiency are highly dependent on the level of dietary methionine intake. Animals develop signs of choline deficiency only when the diet contains no choline and 0.12% or less of methionine.

(3) Two chronic experiments in which rats have been kept on a methionine deficient diet have now been in progress for 14 and 22 months. Results to date have failed to reveal cirrhosis or hepatomas in any of the animals autopsied. Surviving rats will be followed for an additional 6 to 12 months.

Part A (continued)

Significance: Chronic choline deficiency is the only nutritional deficiency that has been found to induce liver cancer in experimental animals. It would be of much interest to determine whether other nutritional deficiencies such as chronic amino acid or protein deficiencies might under certain conditions induce hepatomas in experimental animals.

Proposed Course: Rats placed on different deficient diets will be followed and then sacrificed at varying intervals. Detailed morphologic studies in conjunction with chemical investigation will be conducted.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Sidransky, H., and Baba, T.
Chemical pathology of acute amino acid deficiencies: III. Morphologic and biochemical changes in young rats fed valine- or lysine-devoid diets.
J. Nutrition 70: 463-483, 1960.

2. Sidransky, H.
Chemical pathology of nutritional deficiency induced by certain plant proteins.
J. Nutrition 71: 387-399, 1960.

3. Sidransky, H., and Farber, E.
Liver choline oxidase activity in man and in several species of animals.
Arch. Biochem. Biophys. 87: 129-133, 1960

4. Sidransky, H., and Pearl, M. A.
Pulmonary fungus infections associated with steroid and antibiotic therapy.
Dis. Ches. In press.

5. Pearl, M. A., and Sidransky, H.
Candida endocarditis. Two new cases, with a review of twelve cases previously reported.
Am. Heart J. 60: 345-353, 1960.

Honors and Awards relating to this project:

Member, Society for Experimental Biology and Medicine
Member, American Association for Cancer Research

Serial No. NCI 542
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The histopathogenesis of a peculiar lesion of the juxtaglomerular apparatus and of the glomerulus discovered by us in a patient suffering aldosteronism and hypokalemia

Principal Investigator: Ross C. MacCardle

Other Investigator: Frederic C. Bartter, GMET, NHI, and P. Pronove of NHI

Cooperating Units:

Man Years (calendar year 1960):
Total: 1/8
Professional: 1/8
Other: --

Project Description:

Objectives: We discovered a remarkable renal lesion in a hypokalemic boy producing excess alderstone. The juxtaglomerular apparatus was greatly enlarged and part or all of the glomerulus was found to be in the form of a syncytial sheet of cells, evidently providing a block to the blood flow. The glomerulosa of the cortex of the adrenal gland was hyperplastic. It was difficult to determine whether this was due to excess production of aldosterone, or whether it was a developmental lesion that induced hyperplasia of the adrenal cortex. The focus of production of aldosterone is believed to be the adrenal glomerulosa.

Methods: A microscopic study by conventional histologic methods and special cytological procedures of kidney and adrenal glands of man and of dogs following partial construction of the renal arteries

Major Findings: We have found this juxtaglomerular lesions in two patients with hypokalemia, although it was more marked in the dwarf-boy. The mitochondrial batonets of the distal portion of the uriniferous tubule was rich in K.

Significance: This is a lesion described for the first time and may be related to the early stages of development of an aldosterone-secreting neoplasm of the adrenal cortex. It may be that such hyperplastic and neoplastic growths may be partially corrected.

Part A (continued)

Proposed Course: We intend to try to produce this lesion in dogs by injecting excess aldosterone, or by implanting an aldosterone-producing adrenal cortical tumor.

Part B included: Yes

PHS-NIH
Individual Project Report
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Part B. Honors and Awards and Publications

Publications other than abstracts from this project:

The human renal lesion was described at the First International Endocrinological Congress held in Copenhagen in 1960. A full paper is in preparation.

Serial No. NCI 543
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The cytoplasmic and intranuclear ribonucleic acid and mineral (Ca and Mg) of different protein-secreting plasma-cell tumors of mice.

Principal Investigator: Ross C. MacCardle

Other Investigator: Michael Potter and Karl W. Chapman

Cooperating Units:

Man Years (calendar year 1960):
Total: 1/8
Professional: 1/8
Other: --

Project Description:

Objectives: It was believed to be important to determine whether or not there is any difference in the minerals and RNA of different plasma cell tumors that could be related to the type of protein secreted, and whether this could be influenced by magnesium deficient diet or by injection of ribonuclease into the living tumor bearing mouse. The template hypothesis states that in protoplasm a hypothetical model (template) exists upon which amino acids are deposited to form complex proteins (protein synthesis). It is generally believed that RNA is the template.

Methods: A microscopic study by Brachet's pyronin-methylane green-ribonuclease technique, and by microincineration.

Major Findings: It was found that gamma-globulin secreting tumors are generally rich in cytoplasmic RNA and rich in calcium and magnesium, whereas those secreting alpha and beta globulin are poor in cytoplasmic RNA and rich in intranuclear RNA. The Ca and Mg varies in the latter, being sometimes rich and sometimes poor. The nucleolus of the gamma-globulin tumors is large and rich in silica.

Significance: It may be that the protein synthesis requires a template of calcium and magnesium and possibly silica. Thus the formation of plasma cells may be altered by diets deficient in these elements.

Part A (continued)

Proposed Course: We have already reported these findings at the Paris Congress of Cell Biology and are now reparing a manuscript. We intend to pursue these findings further.

Part B included: No

Serial No. NCI 544
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Experimental studies on the pathogenesis of hepatomas in rats induced by certain chemical carcinogens

Principal Investigator: Herschel Sidransky

Other Investigators: Harold P. Morris, Laboratory of Biochemistry, NCI (Section B (1))

Cooperating Units:

Man Years (calendar year 1960):
Total:
Professional:
Other:

Project Description:

Objectives: To study the morphologic and chemical changes in the course of development of hepatomas in rats ingesting selected chemical carcinogens.

Methods: A. Studies with Ethionine. Ethionine has been reported to induce hepatomas in rats. Although much experimental work has been conducted in the acute actions of ethionine, little is known about its action in chronic experiments leading to hepatomas. Several studies have been started to study certain aspects in the development of hepatomas.

Other workers have reported that ethionine partially inhibits liver regeneration following partial hepatectomy. In one group of experiments we have performed partial hepatectomies on rats and then placed the animals on a purified diet containing ethionine. This was conducted to determine the influence of partial hepatectomy on the development of hepatomas.

Rats ingesting ethionine plus homocystine have been reported to develop earlier and more advanced nodular hyperplasia of the liver than rats ingesting ethionine alone. The mechanism by which homocystine acts to accelerate these morphologic changes is not known. We are now conducting studies designed to learn how homocystine acts in these experiments.

Part A (continued)

P-Hydroxypropiophenone (PHP) has been reported to inhibit the development of liver tumors induced by butter yellow. The effect of PHP on hepatoma induction by the chronic ingestion of ethionine has been investigated.

Ethionine and N-2-fluorenylacetamide have each been found to induce hepatomas in rats. Experiments have been conducted to determine whether these two agents will act synergistically in the development of hepatomas.

B. Studies with N-2-fluorenylacetamide (2-FAA). Many investigators have described a sex difference in incidence of liver tumors in rats induced by the chronic ingestion of 2-FAA. Males have been reported to have a higher incidence than females. In our own study on the influence of dietary intake of pyridoxine on tumorigenesis in rats ingesting 2-FAA we observed certain morphologic differences in the liver tumors of the two sexes. This led us to perform acute experiments in which the sequence of morphologic changes in the livers of male and female rats were studied and chronic experiments concerned with the role of survival and with the dosage of carcinogen on the sex difference in tumor incidence and the morphologic differences of the tumor types.

P-Hydroxypropiophenone (PHP) has been described to inhibit the development of liver tumors caused by butter yellow. Along with the study described previously in which the influence of PHP on ethionine liver tumorigenesis was studied, a study of the influence of PHP on 2-FAA liver tumorigenesis is in progress.

Major Findings: A (1) Chronic experiments in which inbred Osborne-Mendel and Buffalo rats ingest ethionine in the diet are in progress. Osborne-Mendel rats ingesting ethionine for 7 or more months have a 67% incidence of liver tumors after an average survival of 9.7 months. The first hepatomas were observed after 7 months. The liver tumor is transplantable and has now been carried for nine generations. Selected enzymes, catalase, glucose-6-phosphatase and tryptophan perridase, are being studied in this transplantable tumor.

(2) Preliminary studies suggest that rats with partial hepatectomies and ingesting ethionine have a delayed induction period for hepatomas. Hepatomas were first observed after 11-3/4 months. In acute experiments rats with partial hepatectomies and ingesting ethionine have been sacrificed at weekly intervals for 3 weeks and the histologic changes in the liver have been studied. The results indicate that even though liver regeneration is partially inhibited in rats ingesting ethionine in comparison with rats on the control diet, the histologic changes in the liver of the experimental animals are similar to non-hepatectomized animals ingesting ethionine.

Part A (continued)

(3) In acute studies in which young rats were force-fed for 3 days synthetic diets low or devoid of methionine and with low choline, the addition of homocystine improved growth but also appeared to accentuate the signs of acute choline deficiency. This suggested that we should investigate the possible role of choline deficiency on the action of ethionine on the livers of rats in long-term experiments. Chronic experiments are in progress in which rats are being fed diets containing ethionine with different levels of choline with and without homocystine.

(4) In one chronic experiment rats ingesting ethionine plus PHP for 7 to 13 months were found to develop less hepatomas (1 of 9) than rats ingesting ethionine alone for 7 to 17 months (10 of 15).

(5) Experiments in which rats were fed control, ethionine, 2-FAA, and ethionine plus 2-FAA diets have been conducted. Rats fed the ethionine plus 2-FAA diet died with hepatomas at an earlier time than in any of the other groups. In similar experiments in which animals were sacrificed at weekly intervals for 6 weeks, the histologic changes in the liver of rats fed the ethionine plus 2-FAA diet were the most advanced of all groups.

B (1) One publication describes the sex difference in liver tumorigenesis in rats ingesting 2-FAA. When rats were fed a diet containing .03% 2-FAA males were found to have a higher incidence of liver tumors (12 of 12) than did females (5 of 14). Female rats fed .06% 2-FAA developed liver tumors in 6 of 15 animals. Female rats fed a diet containing .03% 2-FAA but in which their average duration of survival was prolonged by removing breast tumors developed a higher incidence of liver tumors (7 of 9). Morphologically the liver tumors in the male rats were less differentiated and more anaplastic than those of the female rats. In acute experiments (4 months) certain histologic changes in the livers of male rats were present earlier and were different from those in the livers of female rats. Testosterone was found to be influential in the development of the early histologic changes in the livers of male rats.

(2) Experiments in which rats were fed a diet containing 2-FAA with and without PHP have been followed for 7 months. Rats will be followed for 6 additional months.

Significance: The present experiments are designed to gain information concerning the morphologic and biochemical changes in the liver of experimental animals exposed to certain chemical carcinogens. Knowledge of the progression of morphologic and chemical changes in the livers of experimental animals in response to certain carcinogens should be of importance in the understanding of liver carcinogenesis.

Part A (continued)

Proposed Course: Rats ingesting diets containing ethionine or 2-FAA will be followed in experiments under different conditions. Animals will be sacrificed at varying intervals and morphologic and chemical studies will be performed on the livers.

(Note: In addition to work described above, collaborative work related to liver carcinogenesis with 2-FAA is being carried out with Dr. H. P. Morris. Details will be given in his report.)

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Sidransky, H., Wagner, B. P., and Morris, H. P.
Sex difference in liver tumorigenesis in rats ingesting N-2-fluorenylacetamide.
J. Nat. Cancer Inst. 26: 1961. In press.

2. Morris, H. P., Sidransky, H., Wagner, B. P., and Dyer, H. M.
Some characteristics of transplantable rat hepatoma No. 5123 induced by ingestion of N-(2-fluorenyl)phthalamic acid.
Cancer Res. 20: 1252-1254, 1960.

Serial No. NCI 545
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS–NIH
Individual Project Report
Calendar Year 1960

Part A.

Project Title: Study of liver catalase and α-levulinic acid dehydrase in mice bearing polyoma virus-induced tumors

Principal Investigator: Clyde J. Dawe

Other Investigators: Donald P. Tschudy, Miroslav Rechcigl, and L. W. Law

Cooperating Units:

Man Years (calendar year 1960):
Total: Absorbed in NCI 521a
Professional:
Other:

Project Description:

Objectives: Previous studies by Drs. Tschudy, Rechcigl and others have shown that the activities of both α-levulinic acid dehydrase and catalase become depressed in the livers, on a weight-unit basis, of mice bearing transplanted tumors. Both of these enzymes contain heme moieties and it has been thought that the observed depression of both in tumor-bearing animals is probably related to a defect in a synthetic pathway that is common to both enzymes.

Because of certain morphologic and biologic peculiarities of polyoma virus-induced tumors of the salivary glands of mice, the question was raised as to whether the response of the liver enzymes would undergo the same changes in these animals as in those previously studied with transplanted tumors.

Methods: Tumors of the salivary glands and of other organs (thymic tumors, subcutaneous fibro-sarcomas, mammary tumors) are induced by inoculation of newborn mice with polyoma virus. The animals are killed and the liver enzyme activities are determined when the tumors reach varying sizes and in some cases when the animals appear moribund. Total body weight, total liver weight, total tumor weight, and hematocrit levels are determined. Complete necropsy is performed and the tumors are classified histologically and the proportion of tumor necrosis is estimated. Evidence of metastasis is also recorded. Attempts are then made to correlate enzyme activity on a total-liver as well as on a per-gram of liver basis with (a) total tumor weight, (b) tumor weight/body weight ratio, (c)

Part A (continued)

hemotocrit values, (d) histologic type and degree of anatomic spread of tumor. Control studies are carried out on non-tumor-bearing mice of comparable age, and on pregnant mice.

Major Findings: This work is still incomplete and much of the data still has to be analyzed. However, there appears to be a significant difference between the effect of large polyoma tumors on liver α-levulinic acid dehydrase (ALAD), and the effect of transplanted tumors of comparable size on the same enzyme. Mice bearing polyoma tumors show little or no depression of ALAD until they become moribund, at which time there is still only a slight or moderate depression of activity. Mice bearing transplanted tumors such as Sarcoma 37, Sarcoma 180, or transplanted leukemias have shown marked depression of ALAD at tumor weights comparable to those of the polyoma tumors. In some mice bearing very large virus-induced tumors, the liver ALAD was even slightly elevated above the control value, whereas it would be expected to be markedly depressed if the tumor were a transplanted one.

With regard to catalase, there appears to be little difference between polyoma-induced tumors and transplanted tumors. In both cases, the catalase activity is not markedly affected until the animal becomes severely anemic and/or moribund.

Significance: The findings seem to provide evidence on two points. First, weight-for-weight, polyoma virus-induced tumors do not affect the liver ALAD of their hosts to the same extent that certain transplanted tumors do. This may merely signify that the transplanted tumors tested represent very unusual tumor "parasites" as a result of their long transplantation histories. Or it may mean that polyoma-virus induced tumors differ from other tumors in some way. Perhaps they are still "dependent" tumors, or have not yet progressed to the point where some product is capable of depressing ALAD.

Secondly, the divergent results with ALAD and catalase suggest that the synthesis, storage, or degradation of these two enzymes may not always run parallel. Somewhere in their synthetic, storage, and degradation patterns there must be a divergence, and apparently the polyoma tumors have their action at a point which is past this point of divergence in one or more of these three aspects that control activity at any given time.

Proposed Course: We wish to complete the collection and analysis of data before outlining extensions of this work. A logical sequel to this study is to determine the liver enzyme changes in mice bearing successive transplant generations of polyoma virus-induced salivary gland tumors. By this means one could determine whether the capability of depressing ALAD and catalase can be "acquired" in the course of time, as a part of the phenomenon of tumor progression. One might also then be able to correlate the acquisition of ALAD and catalase-depressing ability with certain other biologic changes and with morphologic changes.

Part B included: No

Serial No. NCI 546
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Study of the response to rat virus (RV) by rat salivary gland in organ cultures

Principal Investigator: Clyde J. Dawe

Other Investigators: Lawrence Kilham, DBS

Cooperating Units:

Man Years (calendar year 1960):
Total: Absorbed in NCI 521a
Professional:
Other:

Project Description:

Objectives: Rat Virus (RV) was isolated several years ago by Dr. Kilham, from rats bearing transplanted lymphomas, as well as from non-tumor-bearing rats. Dr. Kilham found this virus to be similar to the mouse polyoma virus in the following respects: it was often present in transplanted lymphomas as well as in a high percentage of otherwise normal rats; it was found in highest quantities in salivary glands and kidneys of infected animals; it could be propagated readily in rat embryo tissue cultures; it produced the hemagglutination phenomenon with guinea pig red cells; it did not produce the hemadsorption phenomenon; and it was relatively resistant to heat (80°C for 15 min.) and ether. RV was shown to be distinct from polyoma virus in that it was non-infectious for mice and there were no serologic cross-reactions for the two viruses. Also, it has not been demonstrated that RV induces tumors in any animal.

However, "spontaneous" infection was so universal among rats that it was not possible to test for tumor-inducing action of RV in previously uninfected animals. Because we had previously demonstrated a response of mouse salivary gland tissue to polyoma virus which was similar to the tumor response in vivo, it was thought that this tissue culture system might be of help in determining the response of rat salivary gland to RV in the absence of antibody or other immune factors.

Methods: Submandibular salivary glands from suckling Fischer rats are removed aseptically and cut into 1.0 mm. explants. Half of the explants are infected with RV and the remainder are used as controls. Gelatin sponge matrix cultures are prepared from each group and are carried for varying periods up to 32 days. The results are evaluated by microscopic examination of serial sections and by virus assay of culture fluids by the hemagglutination titration method.

Part A (continued)

Major Findings: Intranuclear inclusion bodies were found in cells of the infected cultures but not in the controls at all time intervals from 10 to 32 days. These inclusion bodies were present in epithelial as well as connective tissue cells, and were similar to those previously found in mouse salivary gland cultures infected with polyoma virus. They were more numerous and tended to be larger, more eosinophilic, and smoother in contour than inclusion bodies associated with polyoma virus.

Certain other changes in the cultures resembled those in the mouse salivary gland-polyoma virus system. There was marked necrosis of the mesenchymal cells, and there was enlargement of epithelial cells and disruption of the acinar pattern. In the RV-infected cultures, as in polyoma virus-infected cultures, there was a marked paucity of macrophages and fibroblasts surviving on the walls of the culture tubes. However, there was not an epithelial proliferative response comparable to that seen in the polyoma virus system. Only occasional mitotic figures were found in the RV-infected cultures, and there was at best only a small amount of cellular penetration of the gelatin sponges adjacent to the explants.

Significance: The observations have revealed additional aspects in which the RV, or the cell response to it, resembles polyoma virus. At the same time, the findings do not show a proliferation-promoting effect (PPE) of the particular RV preparations used that could be compared with the PPE of highly oncogenic preparations of polyoma virus. This situation suggests that some fundamental constitutional character(s), correlating with oncogenic capacity, is lacking in RV. This might be either a qualitative or a quantitative deficiency. The possibility that the PPE of RV might be increased by certain procedures is an interesting one that remains to be proved.

Proposed Course: A report for publication of the above findings is in preparation. Dr. Kilham is attempting to alter the RV in the direction of becoming more oncogenic. If such alteration is achieved, it will then be important to repeat the study described here with the new strain, to see if *in vitro* response correlates with *in vivo* response. The information from the combined studies would be of great assistance in evaluating the organ culture systems as an indirect method of detecting oncogenic action of viral agents.

Part B included: No

Serial No. NCI 501
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland, and New Orleans, Louisiana

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Geographic pathology field studies on urinary bladder cancer in New Orleans, Louisiana

Principal Investigator: Lucia J. Dunham, Harold L. Stewart, and Harold F. Dorn

Other Investigator: Alan S. Rabson

Cooperating Units:

Man Years (calendar year 1960):
Total: 1 5/6
Professional: 1 1/3
Other: 1/2

Project Description:

Objectives: The objective of this project is to complete an extensive questionnaire study of patients with urinary bladder cancer in a southern U.S.A. city with a high incidence of bladder cancer compared to two other southern cities. (Dorn and Cutler, Morbidity from Cancer in the United States, Public Health Monograph No. 56.) The data will be analyzed with a view to identifying factors associated with cases of urinary bladder cancer. Basic data on the incidence and pathologic (microscopic) diagnosis of the cancers are being obtained.

Methods: For the area under study and the period of study all patients possible with the diagnosis of urinary bladder cancer are interviewed, and an equal number of control patients as well. The data recorded include residence, occupation, medical and surgical history, and information about diet and about the use of alcoholic and non-alcoholic beverages and of tobacco. Records are kept of the small number of patients not interviewed, their diagnoses, and the reasons for failure to interview.

A coding statistician and one medical social worker interviewer are currently employed in the project. Interviewing was started in New Orleans on 15 February 1958.

Part A (continued)

Major Findings: Data from about 260 patients and an equal number of control patients have been reviewed and coded. It is estimated that pathologic material will have been examined from about 95 percent of the cancers at the date on which this report is submitted.

Data on environmental factors of patients with cancer of the bladder diagnosed at less than age 50 were compared with the data from patients aged 50 and over, but differences were not considered to be significant. There is a suggestion from the data that the risk of bladder cancer may be excessive among barbers, although this is a preliminary impression only, based on a small number of patients.

Significance: The significance of the study lies in the hope of supplying a sound scientific basis for the understanding of factors in habit or environment as they may be related to a high incidence of urinary bladder cancer.

Proposed Course: It is planned to continue the study for a total of four or more years. Data will be tabulated and analyzed and a report will be prepared for publication.

The possibility is being considered of conducting a survey of morbidity and mortality from cancer in barbers resident in a large U.S.A. city.

Part B included: No

Serial No. NCI 501a
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Geographic pathology field studies on uterine cancer in New York City, Israel, and Washington, D.C.

Principal Investigator: Lucia J. Dunham, Harold F. Dorn, and Harold L. Stewart

Other Investigators: John H. Edgcomb, Louis B. Thomas

Cooperating Units:

Man Years (calendar year 1960):
Total: 5/6
Professional: 1/3
Other: 1/2

Project Description:

Objectives: The objective of this project is to complete an extensive questionnaire study of women with uterine cancers in different geographic areas and racial groups, and to analyze the data obtained with a view to identifying factors suspected of predisposing to or causing female genital cancers. Also, basic data on the incidence and pathologic (microscopic) diagnoses of the cancers are obtained.

Methods: For the area under study and the period of study all patients possible with the diagnosis of uterine cancer are interviewed, and an equal number of control patients as well. The confidential data recorded include social, medical, and surgical history, and menstrual, marital, and pregnancy data. Records are kept of the small number of patients not interviewed, their diagnoses, and the reasons for failure to interview. Pathologic material is studied in all cases.

A coding statistician and one medical social worker interviewer are currently employed in the project.

Major Findings: 1. Data from 3,514 cancer and control patients, Jewish and non-Jewish white, in New York City and Israel have been reviewed, coded, tabulated, and analyzed. Pathologic material has been examined in the cancer cases. A report entitled Epidemiologic Study of Cancer of the Uterus in Jewish and Non-Jewish White Women has been prepared. The authors include, in addition to the National Cancer Institute investigators, Dr. A. Symeonidis, Thessalonika, Greece, and Dr. J. Casper, Petah

Serial No. NCI 501a
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Geographic pathology field studies on uterine cancer in New York City, Israel, and Washington, D.C.

Principal Investigator: Lucia J. Dunham, Harold F. Dorn, and Harold L. Stewart

Other Investigators: John H. Edgcomb, Louis B. Thomas

Cooperating Units:

Man Years (calendar year 1960):
Total: 5/6
Professional: 1/3
Other: 1/2

Project Description:

Objectives: The objective of this project is to complete an extensive questionnaire study of women with uterine cancers in different geographic areas and racial groups, and to analyze the data obtained with a view to identifying factors suspected of predisposing to or causing female genital cancers. Also, basic data on the incidence and pathologic (microscopic) diagnoses of the cancers are obtained.

Methods: For the area under study and the period of study all patients possible with the diagnosis of uterine cancer are interviewed, and an equal number of control patients as well. The confidential data recorded include social, medical, and surgical history, and menstrual, marital, and pregnancy data. Records are kept of the small number of patients not interviewed, their diagnoses, and the reasons for failure to interview. Pathologic material is studied in all cases.

A coding statistician and one medical social worker interviewer are currently employed in the project.

Major Findings: 1. Data from 3,514 cancer and control patients, Jewish and non-Jewish white, in New York City and Israel have been reviewed, coded, tabulated, and analyzed. Pathologic material has been examined in the cancer cases. A report entitled Epidemiologic Study of Cancer of the Uterus in Jewish and Non-Jewish White Women has been prepared. The authors include, in addition to the National Cancer Institute investigators, Dr. A. Symeonidis, Thessalonika, Greece, and Dr. J. Casper, Petah

Part A (continued)

Tiqva, Israel. The manuscript of the report is being revised subsequent to critical comments by the authors and by other investigators. It is planned to publish the report as a U. S. Public Health Service Monograph.

2. Epidemiologic Study of Cancer of the Uterus in Negro Women

a) In New York City. Data from 458 patients with cancer of the uterus and 530 control patients have been reviewed and coded. Tabulation and analysis of data are nearly complete. Pathologic material has been examined in the cancer cases. There are small differences in the environmental factors of patients with cancer of the cervix and control patients. Most of these are not statistically significant.

b) In Washington, D.C. Data from 363 patients with cancer of the uterus and 380 control patients have been reviewed and coded. When tabulated and analyzed, the data will be combined with the data from Negro women in New York City and a report will be prepared for publication.

Significance: The significance of the study lies in the hope of supplying a sound scientific basis for the understanding of factors of custom, habit, or environment as they may be related to relatively high frequencies of uterine cancers in some groups of women as contrasted to intermediate or relatively low frequencies in other groups. In addition, a critical evaluation of the study, prepared at the same time as the report, should provide a useful commentary on techniques in the epidemiology of cancer.

Proposed Course: It is planned to publish the report of the data from Jewish and non-Jewish white patients and to prepare for publication a report of the data from Negro patients. Other methods available for studying the epidemiology of cancer of the uterus will be explored.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Dunham, L. J., Thomas, L. B., Edgcomb, J. H., and Stewart, H. L.: Some environmental factors and the development of uterine cancers in Israel and New York City.
Acta Unio Intern. Contra Cancrum 16: 1689-1692, 1960.

Serial No. NCI 501b
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Geographic Pathology. Experimental studies relating to the geographic pathology of oral cancer

Principal Investigator: Lucia J. Dunham

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: The objective of this project is to examine the mucous membrane of the hamster cheek pouch for proliferative changes that may develop when test substances are chronically maintained in contact with these surfaces. Substances used are environmental materials suspected of being mildly carcinogenic for man.

Methods: The pellets which have been prepared for insertion in the right cheek pouch of the hamster include the tobacco, lime, gambir, areca nut, and pepper leaf of the Far Eastern "betel quid," the plastic material used in preparing "false" teeth, and tobacco tars and control materials.

Major Findings: The operation for inserting pellets in the pouch has been performed on more than 400 animals. No animals survive. Gross and microscopic lesions of the pouch wall have been observed in 24 animals. The lesions were inflammatory except in five instances. Four of the pouches showed epithelial hyperplasia with or without epithelial atypia or hyperkeratosis. There was one squamous papilloma. Lesions were not consistently associated with a particular test substance. Neoplasms can be initiated in the pouch wall by using pellets containing a known carcinogen (see current studies, Serial No. NCI 536, Dr. Katherine Herrold).

Part A (continued)

Significance: The significance is to reach an improved understanding of chronic irritant factors as they may related to cancer of the mouth in man. The epithelium of the hamster cheek pouch is believed to be moderately reactive to environmental stimuli. However, the response of the tissues of the pouch to the substances tested was non-specific.

Proposed Course: A report of the investigation is being prepared for publication. A preliminary study of the results of painting the cheek pouch of infant hamsters with solutions of tobacco tar or a detergent has been started. The effects of pre-treating the mother with urethane and of treating the infant hamster with urethan during the course of pouch painting are being investigated.

Part B included: No

Serial No. NCI 524
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies of the growth of polyoma virus in cultures of P388D$_1$ cells

Principal Investigator: Alan S. Rabson

Other Investigators: Frances Y. Legallais

Cooperating Units:

Man Years (calendar year 1960):
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: To determine the nature and sequence of processes involved in the growth of polyoma virus in P388D$_1$ cells.

Methods: Polyoma virus has been grown and titrated in cultures of P388D$_1$ cells using conventional tissue culture virological methods. Most of the studies have been carried out in cell cultures grown and maintained in medium containing 20% autoclaved nonfat milk; however, growth of the virus is also being studied in P388D$_1$ cells grown in chemically defined protein-free media (NCTC 109 of Evans), and in media containing serum.

Cytochemical studies have been carried out in collaboration with Dr. Robert Love of the Laboratory of Pathology and have involved the use of his toluidine blue-molybdate stain for nucleic acids, the Feulgen reaction for DNA and the alkaline fast green procedure for histones. Fluorescent antibody studies have been carried out in collaboration with Dr. P. T. Wertlake of the Pathologic Anatomy Branch and have utilized immune mouse sera and fluorescein labeled anti mouse gamma globulin prepared in rabbits.

Major Findings: Cytochemical studies of milk-adapted P388D$_1$ cells infected with polyoma virus (carried out in collaboration with Dr. Robert Love, Laboratory of Pathology) have shown a series of changes first in the nucleus and terminally in the cytoplasm. The earliest abnormality consists of enlargement of the nucleus, nucleolus and nucleolinus, and the

Part A (continued)

appearance of intranuclear vacuoles in which normal chromatin and ribonucleoprotein (RNP) are replaced by abnormal histone-free deoxyribonucleoprotein (DNP). This DNP then disappears and DNP inclusions form in the vacuoles. The RNP of the pars amorpha of one or more of the enlarged nucleoli diffuses, fills the vacuoles and becomes incorporated into the inclusion material. As the RNP increases in the inclusions, they become less Feulgen positive. Later the RNP that was derived from the nucleolus disappears from the vacuoles and the inclusions, and a new type of RNP appears in the inclusions which are then Feulgen negative. The presence of both DNA and RNA in these inclusions was demonstrated by the toluidine blue-molybdate stain with appropriate nuclease digestion procedures. The inclusions contained histone-free protein throughout their development. The fluorescent antibody studies have demonstrated both diffuse and particulate viral antigen in the nuclei of infected cells, and the precise relationship of the antigenic material to the inclusion bodies observed in the cytochemical studies is being investigated.

Significance: Understanding of the cytological and cytochemical processes involved in the replication of polyoma virus is of importance in the general problem of cellular nucleic acid and protein synthesis, and may have importance in the study of the mechanisms involved in viral carcinogenesis.

Proposed Course: The relationship of the cytochemical observations to the appearance of infectious virus in the cells is being investigated by infectivity titrations of intracellular and extracellular virus at various stages in the cytopathic process. The fluorescent antibody studies will be continued and we will attempt to determine the relationship of the inclusion bodies to the appearance of viral antigens. We also plan to study the cytological and cytochemical changes in P388D_1 cells during growth of different strains of polyoma virus to determine whether there are any differences in cytopathology associated with differing oncogenic potency of the strains.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Love, R., and Rabson, A. S.
   Cytochemistry of Nucleic Acids in Murine Lymphoma Cells Infected with Polyoma Virus
   Nature. In press.

2. Love, R., and Rabson, A. S.
   Nucleoproteins in Murine Lymphoma Cells Infected with Polyoma Virus *in vitro*
   Pathologie et Biologie. In press

Serial No. NCI 531
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Attempts to isolate oncogenic viruses from human tumors

Principal Investigator: Alan S. Rabson

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To isolate oncogenic viruses from human tumors.

Methods: Extracts of fresh human tumors are prepared and inoculated into cultures of human cells, and into newborn and fetal Syrian hamsters. The tissue culture fluids are passaged to newborn and fetal hamsters at intervals.

Major Findings: Preparations from 45 human tumors have been studied thus far. In 1960, materials from patients with synovial sarcomas, lymphosarcomas, lymphocytic leukemias, Wilm's tumors, papillary bladder tumors, embryonal rhabdomyosarcomas, Hodgkin's disease, and hemangioma of the parotid gland have been studied.

Although a few of the animals inoculated with tumor extracts have subsequently developed tumors, there is no evidence that these tumors were related to the inoculum. In the tissue culture studies, a cytopathic agent has been isolated from an extract of a human laryngeal carcinoma using cultures of a strain of human cells grown in milk medium. The agent has been adapted to grow in HeLa cell cultures where it also causes a cytopathic effect. The agent has been inoculated into newborn mice and Syrian hamsters and has no obvious pathogenicity in these animals. We are now attempting to identify it serologically and to determine whether it produces proliferative effects in organ cultures of human respiratory mucosa.

Part A (continued)

Significance: The demonstration of a human tumor virus would be of great importance in studies of human cancer.

Proposed Course: The inoculation studies with extracts of selected human tumors using the tissue culture systems and animal inoculation systems described above will be continued. Attempts to identify the agent isolated from the human laryngeal tumor and to determine whether it has any proliferative effect on human respiratory mucosa will be continued.

Part B included: No

Serial No. NCI 532
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies of lung tumors produced by intratracheal inoculation of polyoma virus in Syrian hamsters

Principal Investigator: Alan S. Rabson

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total:
Professional:
Other:

Project Description:

Objectives: To study the pathology and pathogenesis of lung tumors produced by intratracheal inoculation of polyoma virus, and to attempt to determine the role of the virus in the induction of the tumors.

Methods: Weanling male hamsters were used in the studies. The strain of polyoma virus was initially isolated in cultures of milk-adapted $P388D_1$ cells from a cell-free filtrate of a parotid tumor extract from a C3H/Bi mouse. Virus pools from the 10th and 14th consecutive passages of the virus in $P388D_1$ cells were used for intratracheal inoculation.

Animals were anesthetized and their tracheas were exposed by midline neck incisions. One ml. of virus preparation or control fluid was injected directly into the tracheal lumen. Twenty-two animals were inoculated with virus preparations and 14 with control fluids.

Major Findings: Of the 22 hamsters inoculated intratracheally with polyoma virus, 16 died during a 400-day period of observation and all of these were autopsied. Proliferative lesions of bronchiolar and alveolar lining cells were found in the lungs of 5 animals, and lung tumors with histologic features of bronchiolar carcinoma, alveolar cell carcinoma and squamous cell carcinoma were found in 6 of the 16. The areas of squamous cell carcinoma appeared to arise in bronchioles and intra-pulmonary bronchi; however, no evidence of origin from trachea or main bronchi was found. A transplantable squamous cell carcinoma has been derived from one of the lung tumors and is now in its 16th transplant generation. Attempts to isolate polyoma virus from one of the primary lung tumors and from the transplanted squamous cell carcinoma have been unsuccessful.

Part A (continued)

Significance: Since most of the studies of etiology of lung cancer in laboratory animals and man have been concerned with carcinogenic hydrocarbons and metals, the demonstration of tumor induction after intratracheal inoculation of an oncogenic virus is of interest. The role of the virus in the production of the tumors, however, is not clear. The failure to demonstrate virus in the one lung tumor tested and in the transplanted tumor is in accord with reports of other investigators on the difficulties encountered in recovering polyoma virus from sarcomatous tumors in hamsters. It is possible that the virus may transform a normal cell into a tumor cell and become integrated into the tumor cell in a manner like that found in lysogenic bacterial cultures. The polyoma virus may thus exist as a provirus and it may be possible to demonstrate it by using techniques employed in bacteriophage studies for induction. If suitable methods can be devised for recovering polyoma virus from the lung tumors of the hamsters, such methods may also be useful for the demonstration of oncogenic viruses in human lung carcinomas.

Proposed Course: Efforts to demonstrate polyoma virus in the lung tumors and in the transplanted squamous cell carcinoma will be continued. Effects of ultraviolet and ionizing radiations, chemical carcinogens and other inducing agents on the tumor cells will be studied.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Rabson, A. S., Branigan, W. J., and Legallais, F. Y.
Lung tumors produced by intratracheal inoculation of polyoma virus in Syrian hamsters
J. Nat. Cancer Inst. 25: 937-966, 1960

Serial No. NCI 547
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Production of tumors in Rattus (Mastomys) natalensis by polyoma virus

Principal Investigator: Alan S. Rabson

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total:
Professional:
Other:

Project Description:

Objectives: To determine the response of Rattus (Mastomys) natalensis to inoculation of polyoma virus.

Methods: Pregnant Mastomys were obtained from the colony maintained in the Laboratory of Pathology by Dr. Harold L. Stewart and Dr. Katharine C. Snell. Newborn Mastomys, less than 12 hours old, were inoculated subcutaneously with polyoma virus grown in milk-adapted $P388D_1$ cells.

Major Findings: Thirty animals from three litters were inoculated with virus, and six months later 24 of the 30 animals were dead. Because of cannibalism, it was not possible to autopsy seven of these 24. Tumors were found in 14 of the 17 animals autopsied. Thirteen animals had renal sarcomas, 2 had sarcomas in the heart, 2 had subcutaneous sarcomas, and 3 had angiomatous liver tumors. No tumors were found in animals inoculated on the day of birth with control tissue culture fluid.

It was possible to isolate polyoma virus in tissue culture from suspension of one of the renal sarcomas of a Mastomys.

Significance: Rattus (Mastomys) natalensis, the multi-mammate mouse is in African rodent intermediate in size between the rat and the mouse. These animals have been used in microbiological studies because they are highly susceptible to infection by plague bacilli, Schistosoma mansoni and Borrelia duttonii. They have also been used in cancer research laboratories following Oettle's report of a high incidence of spontaneous adenocarcinoma of the stomach in a Mastomys colony in South Africa. The response of these animals to infection by a tumor virus is therefore of interest.

Part A (continued)

Since some difference of opinion exists whether to group Mastomys taxonomically with the rat (genus Rattus Fischer) or with the mouse (genus Mus L.), the types of tumors produced by polyoma virus in Mastomys are of interest. The renal sarcomas are grossly and histologically similar to tumors produced by polyoma virus in kidneys of rats and mice. The sarcomas of the heart, however, have not been described in either rats or mice, but are very frequently produced by polyoma virus in Syrian hamsters. Thus far no parotid gland tumors or thymic tumors similar to those produced by polyoma virus in mice have been found in Mastomys. It should be noted, however, that the virus pools we have used show very little oncogenic activity in mice, although they readily produce tumors in Syrian hamsters.

Proposed Course: Studies of Mastomys inoculated with various strains of polyoma virus some of which possess high degrees of oncogenic activity in mice (obtained from Dr. C. J. Dawe, Laboratory of Pathology) to determine whether it is possible to obtain parotid gland tumors or thymic tumors in Mastomys.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Rabson, A. S., Branigan, W. J., and Legallais, F. Y.
Production of tumors in Rattus (Mastomys) natalensis by polyoma virus
Nature 187: 423-425, 1960

Serial No. NCI 517
1. Laboratory of Pathology
2. Pathological Technology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Pathological Technology Section

Principal Investigator: Joseph M. Albrecht

Other Investigators:

Cooperating Units:

Man Years (calendar year 1960):
Total: 30
Professional: 3
Other: 27

Project Description:

Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for all the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition develops and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.

November 1, 1959 - October 31, 1960

Number of investigators	75
Number of pieces of tissue	177,748
Number of bottles of tissue	28,170
Number of blocks cut	98,330
Number of blocks cut serially	2,610
Number of frozen blocks cut	1,276
Number of blocks recut	1,275
Number of slides stained H & E	129,068
Number of slides stained special	29,994
Number of unstained slides	11,516

Photographic Service rendered to the Laboratory of Pathology, November 1, 1959, to October 31, 1960:

Gross

Black & White	815
Color	705

Part A (continued)

Micros	
Black & White	745
Color	502
Lantern Slides	
Black & White	308
Color	876
Prints	
4 x 5	3548
8 x 10	183
Mounted	348

Part B included: No

Serial No. NCI 852
1. Pathologic Anatomy Branch
2. Cytodiagnostic Service
3. Bethesda 14, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

PART A.

Project Title: Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems

Principal Investigator: Dr. Richard A. Malmgren

Other Investigators: Dr. Elizabeth W. Chu

Cooperating Units: None

Man Years (calendar year 1960):
Total: 7
Professional: 4
Other: 3

Project Description:

Objectives:

The primary responsibility of the Cytodiagnostic Service is to provide the staff of the Clinical Center with an accurate and complete exfoliative cytology service. In addition, the Cytodiagnostic Service collaborates in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patient, the course and natural history of the cancer lesion, and the anatomical and physiological changes in the human body associated with various pathologic conditions. The accomplishment of these objectives necessitates the continual improvement in techniques and the origination of new cytologic methods in the laboratory.

Methods Employed:

The standard Papanicolaou staining procedure is applied to preparations of various human specimens. These specimens are then screened and evaluated for morphologic changes in the cellular elements. Special procedures involving the use of fluorescent microscopy, millipore filtration, and various special staining techniques are required in many instances. The principal investigator spent 3 months in Professor Torbjörn Caspersson's laboratory at the Karolinska Institute, Stockholm, Sweden, participating in the application of microspectrophotometric techniques to the study of carcinoma-in-situ and invasive carcinoma of the uterine cervix.

Patient Material:

The figures compiled below are for the last twelve months (12).

Type of material examined	Accessions	# Slides
Wound washings, blood and drainage	1009	4389
Bronchial washings and sputa	177	566
Vaginal and cervical	1345	3472
Miscellaneous Fluids (gastric, prostatic, breast, pleural, etc.)	420	1333
Improvement techniques	104	401
TOTAL	3055	10,161

Institute	Accessions	# Slides
NCI	1930	7186
NHI	176	435
NIAMD	234	708
NIAID	104	328
NINDB	168	538
NIMH	3	12
NIDR	22	72
Referred	416	882

Major Findings:

1. The major contribution of the Cytodiagnostic Service to the diagnostic facilities of the clinical staff has been the early diagnosis of neoplasms of the cervix and lung and of other sites not previously diagnosed. In several instances, exfoliative cytology provided the only tissue confirmation of neoplastic disease before death of the patient.

2. The quantitative evaluation of wound washings, submitted by the Surgery Branch, NCI has been continued with the demonstration of neoplastic cells in many of the washings. In addition, the fluid draining from wounds in the first few postoperative days is being processed and examined for tumor cells and in a number of cases cancer cells have been identified.

3. In collaboration with the Surgery Branch, NCI and the U.S. Public Health Service Hospital, Baltimore, approximately 1600 blood specimens will have been collected this year from cancer patients for examination for cancer cells. Cytologic examination of this material by the streptolysin O millipore technique indicates that cancer cells are more often found in cases of non-resectable cancer than in resectable cases. The cells are more frequently found in local blood from the tumor site than from peripheral blood. In a number of patients in which cancer cells have been found in the local blood there has been no recurrence of the disease to date following surgical treatment.

Major Findings (Continued):

4. The application of the millipore filtration technique to various extra vaginal cytology specimens has increased the efficiency and decreased the time required to process the specimen. It has also permitted a diagnosis to be made which would have been less likely by the older processing methods. Examination of spinal fluid specimens from patients with leukemia using this method has demonstrated leukemic cells in the spinal fluid. The correlation between these observations and the presence of central nervous system involvement is to be determined.

5. In the limited study carried out in Stockholm with microspectrophotometric methods, no correlation was demonstrated between the cell mass, nuclear DNA or nuclear cytoplasmic ratio in the cells from invasive and carcinoma-in-situ cervical cancer patients.

Significance of the Program:

1. The diagnostic value of exfoliative cytology in the clinical evaluation of cancer and its early detection is readily apparent from the activities of the Cytodiagnostic Service in the Clinical Center and throughout the country.

2. The wound washing program contributes to a better understanding of the factors involved in tumor growth, progression and metastases and the reaction of the individual to his neoplasm. In addition, the application of this information to cancer chemotherapy may be of value. Of interest however, is the lack of correlation between the finding of cancer cells in the wound washings and the incidence of recurrence. This has been verified by relatively long term follow-up observations.

3. The study of tumor cells in the blood presents the potential of gaining a better understanding of the factors involved in metastasis. It may be of value in studying certain types of therapy of the neoplastic diseases and diagnosis. Preliminary observations suggest there is little correlation between the finding of tumor cells in the blood and prognosis. Such an observation, as in the case of the wound washings, suggest that factors other than the mere presence of cancer cells are involved in determining metastasis development.

4. The development of new cytologic techniques and the acquisition of specimens from different sites in various diseases continues to enhance the value of the exfoliative cytology procedure as a diagnostic aid. The value of the millipore filter technique is readily apparent and it is anticipated that this procedure will replace the smear preparations in many cytologic laboratories. The use of this method in the study of spinal fluid may provide a useful tool for the evaluation of the involvement of the central nervous system by leukemia and other neoplastic processes.

Proposed Course of the Project:

1. The continuation of a complete and adequate cytologic service for the staff of the Clinical Center is our primary purpose. Continued collaboration with the Clinical Center staff in various research projects and the development of new and improved techniques within the laboratory are an important part of the future program.

2. A continuation of the wound washing, wound drainage, and blood study by quantitative techniques will be continued in collaboration with the Surgery Branch in order to gain a better understanding of the metastasis problem and post surgical recurrence of cancer.

Part B included Yes (x) No ( )

Part B.

Publications other than abstracts from this project:

Borelli, J.I., Ward, J.B., DeWitt, S.H., Del Vecchio, P.R. and Malmgren, R.A. A Starlike Pattern Observed in Cervical Mucus Smears Obtained from Patients with Breast Cancer and Endocrine Disorders. JNCI 25: 1-11, 1960.

Potter, J.F., Longenbaugh, G., Chu, E., Dillon, J., Romsdahl, M., and Malmgren, R.A. The Relationship of Tumor Type and Resectability to the Incidence of Cancer Cells in Blood. Surgery, Gyn. and Ob. 110: 734-738, 1960.

Chu, E.W., and Salmon, M.J. Observations on Vaginal Cornification in Patients Resident in a Mental Hospital. Am. J. Obs. and Gyn. (In Press).

Romsdahl, M.M., Chu, E.W., Hume, R., and Smith, R.R. The Time of Metastasis as Determined in an Experimental System. Cancer (In Press).

Romsdahl, M.M., Chu, E.W., Hume, R., and Smith, R.R. Method for the Cytological Detection of Tumor Cells in Mouse Blood (In Vitro). JNCI (In Press).

Serial No. NCI 854
1. Pathologic Anatomy Branch
2. Cytodiagnostic Service
3. Bethesda 14, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

PART A.

Project Title: Experimental Studies on the Cytopathological Development of Induced Tumors in the Female Genital Tract of Syrian Hamsters

Principal Investigator: Dr. Elizabeth W. Chu

Other Investigators: Dr. Katherine McD. Herrold

Cooperating Units: None

Man Years (calendar year 1960):
Total: 1
Professional: 1
Other:

Project Description:

Objectives:

1. To establish the effects of various carcinogens on the female genital organs of the Syrian Hamsters.
2. To ascertain the histopathological stages by exfoliative cytological methods.
3. To note the time of development of carcinoma-in-situ and to determine its regression or progression to invasive carcinoma.

Methods Employed:

1. Painting of hamster cervices with polycyclic hydrocarbons and tobacco tar at regular bi-weekly intervals. The materials used are all of 1% concentration in commercial acetone.
2. Vaginal smears prior to painting are prepared according to the standard Papanicolaou methods as applied to the human specimens.
3. Histological sections are made to correlate with significant cytological findings.
4. The above methods are applied to both virgin and breeding female Syrian hamsters.

Major Findings:

1. The hamsters that were painted with DMBA (9, 10, dimethyl; 1 , 2,

Major Findings (continued)

benzanthracene) showed atypical cytological changes in the vaginal smears after 1-1/2 months' painting and definite carcinomatous changes in 2 months. These findings were borne out by histological sections.

2. 3,4, Benzpyrene painted cervices showed comparable cytohistological findings after a much more prolonged period than the above group.

3. Tobacco tar in acetone (equal parts) appears to have a mild effect when painted on the cervices. No definite malignant cytological changes have been noted on their vaginal smears after 12 months' painting.

4. The control group using acetone showed atypical cytological changes but no definite evidence of malignant changes.

Significance of the Program:

1. To establish the various developmental stages of carcinoma by cytological methods in an animal system.

2. Most of the studies on the carcinogenic properties of tobacco tar have utilized the skin as the target organ. The advantages for using the uterine cervix are that it helps to eliminate the toxicity of tobacco tar, is easily accessible and histologically is a mucous membrane.

3. The response of Syrian Hamsters to different polycyclic hydrocarbons.

Proposed Course of the Project:

1. All but the tobacco tar painted group of hamsters have been sacrificed after nearly a year's study. During the next year, work will be done to reproduce the various effects noted. Undiluted tobacco tar will be used instead of the half strength one.

2. Comparable work will also be done on the genital organs other than the cervices. The principal investigator also proposes to study the hormonal effects with the development of genital carcinomas.

Part B included Yes (x) No ( )

Part B.

Publications other than abstracts from this project:

Potter, J.F., Longenbaugh, G., Chu, E., Dillon, J., Romsdahl, M., and Malmgren, R.A. The Relationship of Tumor Type and Resectability to the Incidence of Cancer Cells in Blood. Surgery, Gyn. and Ob. 110: 734-738, 1960.

Chu, E.W., and Salmon, M.J. Observations on Vaginal Cornification in Patients Resident in a Mental Hospital. Am. J. Obs. and Gyn. (In Press).

Romsdahl, M.M., Chu, E.W., Hume, R., and Smith, R.R. The Time of Metastasis as Determined in an Experimental System. Cancer (In Press).

Romsdahl, M.M., Chu, E.W., Hume, R., and Smith, R.R. Method for the Cytological Detection of Tumor Cells in Mouse Blood (In Vitro). JNCI (In Press).

Romsdahl, Marvin M., Potter, John F., Malmgren, R.A., Chu, Elizabeth W., Brindley, Clyde O., and Smith, R.R. A Clinical Study of Circulating Tumor Cells in Malignant Melanoma. Surgery, Gyn. and Ob. Dec. 1960.

PHS-NIH
Individual Project Report
Calendar Year 1960

Serial No. NCI 853
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda 14, Maryland

Part A.

Project Title: The Department of Pathologic Anatomy (Pathologic Anatomy Branch, NCI) has as its principal duty the responsibility to serve in investigative efforts of the N.I.H. staff responsible for patient care. In many instances, investigative study of pathological material is of great importance to clinical research.

Staff:

* Dr. Harold L. Stewart, Chief, Pathologic Anatomy Branch
* Dr. Louis B. Thomas, Head, Surgical Pathology and Postmortem Service
* Dr. Richard A. Malmgren, Head, Cytodiagnosis Service
* Dr. John H. Edgcomb, Pathologic Anatomy Branch
* Dr. Alan S. Rabson, Pathologic Anatomy Branch
* Dr. Gregory T. O'Conor, Pathologic Anatomy Branch
* Dr. Elizabeth W. Chu, Pathologic Anatomy Branch
\+ Dr. Peter D. Olch, Resident, Pathologic Anatomy Branch
\+ Dr. Andrew S. Tegeris, Resident, Pathologic Anatomy Branch
\+ Dr. Paul T. Wertlake, Resident, Pathologic Anatomy Branch
\+ Dr. Jack T. Lasersohn, Resident, Pathologic Anatomy Branch
\+ Dr. William C. Roberts, Resident, Pathologic Anatomy Branch
\+ Dr. Seymour M. Sabesin, Resident, Pathologic Anatomy Branch
° Dr. Leon Sokoloff, Laboratory of Pathology and Histochemistry, NIAMD
° Dr. Harold R. Stanley, Oral Pathologist, Clinical Investigations, NIDR
° Dr. Igor Klatzo, Head, Clinical Neuropathology Section, NINDB
° Dr. Herschel Sidransky, Laboratory of Pathology, NCI

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* - These Pathologists constitute the staff of the Department of Pathologic Anatomy, Clinical Center (Pathologic Anatomy Branch, NCI) and spend a major portion of their time in the service and diagnostic work of the Department.

\+ - These Physicians are full time Residents in the Department of Pathologic Anatomy.

° - These associate pathologists spend part time in the activities of the Department of Pathologic Anatomy. In addition, several pathologists on the staff of the Laboratory of Pathology, NCI; i.e. Drs. Swarm, Dawe, Banfield, O'Gara and Stanton, are available for consultation with the staff of the Department of Pathologic Anatomy.

Project Description:

The service functions of the Department during the twelve month period, November 1959 through October 1960 included:

a) 276 autopsy examinations. On page 4 there is a table which shows the number of autopsies performed for each Institute. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held by the staff and residents each morning at 8:30. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the Department participates.

b) 2521 specimens were accessioned in the Surgical Pathology Section. A complete listing of these specimens, by Institute, is given in the accompanying table. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissues removed for specific research purposes.

c) Cytodiagnosis Service: The report for this service is submitted separately.

The objectives of the Department of Pathologic Anatomy in the Clinical Center are twofold: first, to furnish a diagnostic service and second, to aid from a morphological standpoint the various clinical research problems which are under study in the Clinical Center. In the first 2 or 3 years after the Department was established, most of the time of the staff was taken up by diagnostic responsibilities. During the past several years, the staff has been able to spend more time in collaborative research on Clinical Center patients with various members of the Clinical Staff.

Standard methods are used in describing, fixing and sectioning various human organs and tissues and in addition a wide variety of special staining procedures are used.

Distribution of human organs and tissues:

At present there are approximately 45 scientists in the various laboratories at N.I.H. who have requested particular tissues from surgical and postmortem services. On many occasions it is possible to furnish these investigators with fresh human material. A list of these investigators is kept by the secretarial staff and telephone calls are made to the various investigators whenever surgical or postmortem tissues are available.

Conferences:

The staff and residents take an active part in numerous Clinical Center and Institute conferences. In addition, there are several conferences regularly held in the Department including a surgical pathology conference, brain cutting conference and joint pathologists conference.

Part B included Yes (x) No ( )

NCI 853

REPORT OF PATHOLOGIC ANATOMY DEPARTMENT

INSTITUTE AND TYPE OF PATIENT

	NCI		NHI		NIAMD		NIAID		NINDB		NIMH		NIDR		REF		TOTAL	
	IN	FU	IN	FU	IN	FU	IN	FU	IN	FU	IN	FU	IN	FU	IN	FU	IN	FU
Postmortem-Pathologic Anatomy Service																		
# of autopsies performed	186		46		12		15		11						6		276	
Blocks of tissue	10833		3368		836		864		745						189		16835	
Slides	24626		7439		2184		2139		1738						753		38879	
Slides actually stained	14834		3985		1083		1066		953						344		22265	
Surgical Pathology																		
Tissue Specimens	1511		274		271		236		71				133		25		2521	
Slides H-E staining	6557		1188		1474		713		284				210		98		10524	
Frozen Sections	157		68		44				1								270	
Special Procedures	1070		390		320		424		61				10		50		2325	

Part B.

List of Publications and Collaborative Investigative Projects:

A. Dr. Louis B. Thomas:

1. Shaw, Richard K., Moore, Edward W., Freireich, Emil J., and Thomas, Louis B. Meningeal Leukemia. A syndrome resulting from increased intracranial pressure in patients with acute leukemia. Neurology 10: No. 9, Sept. 1960.

2. Chirigos, Michael A., Thomas, Louis B., Humphreys, Stewart R., and Goldin, Abraham. Intracerebral Growth and Treatment of Leukemia L1210. Proc. of Soc. for Exper. Biol. & Medicine 104: p. 643-645, 1960.

3. McFarland, James A., Thomas, Louis B., Gilbert, Joseph W., and Morrow, Andrew G. Myocardial Necrosis Following Elective Cardiac Arrest Induced with Potassium Citrate. J. of Thoracic and Cardiovascular Surgery 40: p. 200, August 1960.

4. Clinical Center Staff Conference at National Institutes of Health. Moderator: Von Sallmann, Ludwig. Discussants: Kaufman, Herbert E., Haase, Gunter, Bartter, Frederick C., and Thomas, Louis B. Primary Amyloidosis. Annals of Int. Med. 52: No. 3, March 1960.

5. Freireich, Emil J., Thomas, Louis B., Frei, Emil III, Fritz, R.D., and Forkner, C.E. A Distinctive Type of Intracerebral Hemorrhage Associated with "Blastic Crisis" in Patients with Leukemia. Cancer 13: p. 146-154, Jan.-Feb. 1960.

6. Moore, Edward W., Thomas, Louis B., Shaw, Richard K., and Freireich, Emil J. The Central Nervous System in Acute Leukemia. A.M.A. Arch. of Int. Med. 105: p. 451-468, 1960.

7. Lipsett, M.B., Dreifuss, F.E., and Thomas, Louis B. Hypothalamic Syndrome Following Varicella. To be published in Amer. J. Med.

8. Current Projects which will lead to Publications:

   1. Cancer of the Uterus. Dunham, Lucia J., Casper, Julian, Dorn, Harold F., Thomas, Louis B., Edgcomb, John H., Symeonidis, Alexander and Stewart, Harold L.

   2. Study of Clinical Center Patients with Carcinoma of Cervix Treated by Pelvic Exenteration with Dr. Robert Smith, Dr. John Dillon and Dr. Jack T. Lasersohn.

   3. Clinical Pathologic Study of Epidermoid Carcinoma of the Head and Neck. Hoye, Robert C., Herrold, K.M., Smith, Robert and Thomas, Louis B.

List of Publications and Collaborative Investigative Projects (Continued):

4. Epidemilogic and Pathologic Studies of Carcinoma of the Cervix in Connecticut and Southwest Region of England. Bailar, John, Haenszel, William, Thomson, Andrew D. (Bland-Sutton Institute of Pathology, Middlesex Hospital, London) and Thomas, Louis B.

9. Exhibit:
   1. Part of exhibit selected by N.I.H. entitled "Central Nervous System Complications of Acute Leukemia." This exhibit is property of Mr. Francis Olson of the Information Unit and was shown at the A.M.A. meetings in Washington, D.C. 11/28/60 - 12/1/60.

B. Dr. John H. Edgcomb:

See separately submitted Annual Report.

C. Dr. Richard A. Malmgren: See separate report for Cytodiagnosis Service.

D. Dr. Alan S. Rabson:

1. Rabson, Alan S., Branigan, William J., and Legallais, Frances Y. Production of Tumours in Rattus (Mastomys) Natelensis by Polyoma Virus. Nature 187: p. 423-425, July 30, 1960. No. 4735.

2. Rabson, Alan S., and Kirschstein, Ruth L. Intracranial Sarcomas Produced by Polyoma Virus in Syrian Hamsters. A.M.A. Arch. of Path. 69: p. 663-671, June 1960.

3. Rabson, Alan S., Branigan, William J., and Legallais, Frances Y. Lung Tumors Produced by Intratracheal Inoculation of Polyoma Virus in Syrian Hamsters. J.N.C.I. (In Press).

4. Love, Robert, and Rabson, Alan S. Nucleoproteins in Murine Lymphoma Cells Infected with Polyoma Virus in vitro. Pathologie et Biologie (In Press).

5. Love, Robert, and Rabson, Alan S. Cytochemistry of Nucleic Acids in Murine Lymphoma Cells Infected with Polyoma Virus. Nature (In Press).

6. Malmgren, R.A., Rabotti, G., and Rabson, A.S. Intracellular localization of Polyoma Virus Antigen Demonstrated with Fluorescent Labeled Antiserums. J.N.C.I. 24: p. 581-588, 1960.

7. Herrold, Katherine M., and Rabson, Alan S. Aberrant Parathyroid Gland in Pharyngeal Submucosa. Arch. of Path. (In Press).

List of Publications and Collaborative Investigative Projects (Continued):

E. Dr. Thomas F. Dutcher:

1. Dutcher, Thomas F., and Fahey, John L. Immunocytochemical Demonstration of Intranuclear Localization of 18S Gamma Macroglobulin in Macroglobulinemia of Waldenström. Soc. for Exper. Biol. & Med. 103: p. 452-455, 1960.

F. Dr. Rowan C. Boylan:

1. Boylan, Rowan C., and Sokoloff, Leon. Vascular Lesions in Dermatomyositis. Arthritis & Rheumatism. 3: No. 5, p. 379-386, Oct. 1960.

G. Dr. Edward B. Price, Jr.:

1. Price, Edward B., Jr. and Ober, William, with others are collaborating in a study of the pathologic aspects of choriocarcinoma and choriadenoma destruens.

2. Fritz, Richard D., Frei, Emil, Price, Edward B., Jr., and Moore, Edward. The Kidney in Acute Leukemia.

H. Dr. William C. Roberts:

1. Familial myocardial disease with Dr. Eugene Braunwald.

2. Absent aortic arch - case reports with Dr. Eugene Braunwald.

3. Focal "embolic" glomerulonephritis with Dr. Alan S. Rabson.

4. Mumps myocarditis - case report with Dr. Samuel Fox.

5. Cystic fibrosis.

I. Dr. Paul T. Wertlake:

1. Protein losing gastroenteropathies with Dr. Robert Gordon.

2. Cell cultures and studies for polyoma virus with Dr. Alan S. Rabson.

J. Dr. Andrew S. Tegeris:

1. Cancer Chemotherapy Correlative Study with Dr. Louis B. Thomas.

2. Cerebellar Meningioma with Low CSF Sugar - case report with Dr. M.W. Brandriss.

3. Aberrant parotid gland tumor - case report with Dr. J.W. Bains.

List of Publications and Collaborative Investigative Projects (Continued):

K. Dr. Jack T. Lasersohn:

1. Cancer of cervix - surgical and pathologic factors with Drs. Louis B. Thomas, Robert Smith and John Dillon.

2. Mucosis fungoides infiltration of the heart.

3. Mephyton Nephritis, fat emboli in kidneys of patients treated with mephyton with Dr. Alan S. Rabson.

4. Case report of patient with positive LE test and recovery from acute hepatitis with Drs. Steven Schenker, John Nasou.

L. Dr. Peter Olch:

1. Chapter on History of Surgery, written for textbook "Principles and Practice of Surgery" by Allen, Harkins, Moyer, and Rhoads -- to appear in 1961 edition.

2. Chapter on History of Gastric Surgery, being written for textbook on Surgery of Gastric and Duodenal Ulcer by Harkins, Nyhus, et al. To appear in 1961.

3. A review of leukemic cases in N.I.H. Pathology Department for presence of pneumocystic carinii infection of lungs.

4. Further studies of factors effecting metastases in the experimental animal.

5. Review of histology of lesions treated with combined Actinomycin D+ radiotherapy.

M. Dr. Seymour M. Sabesin:

1. Investigation of hypersensitivity reactions with electron microscope with Dr. William Banfield.

2. Electron microscope study of Islet cell tumours with Dr. William Banfield.

3. Fluorescent microscope studies on site of formation of Bence-Jones protein and myeloma proteins with Drs. J. Fahey and R. Malmgren.

4. Clinical Papers:

1. Aspergillosis and mucor with Dr. Hirschel Sidransky.

List of Publications and Collaborative Investigative Projects (Continued):

Clinical Papers (Continued):

2. Islet cell adenomas with Dr. John Potter.

3. Investigation of Etheron sponges with Dr. M. Arons.

4. Calcification in lung of leukemias with Dr. John Edgcomb.

5. Neuroblastoma case report with Dr. Louis B. Thomas.

N. Dr. Martin Hicklin:

1. Clinical Center Staff Conference at N.I.H. Moderator: Bartter, Frederick C. Discussants: Liddle, Grant W., Bell, Norman H., Braunwald, Eugene, Hilbish, T.F., Cornell, William, and Hicklin, Martin. Annals of Int. Med. 52: No. 6, p. 1289-1309, June, 1960.

Serial No. NCI-904
1. Laboratory of Physiology
2. Office of the Chief
3. Department of Biology
Princeton University
Princeton, N. J.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The Mechanism of Carcinogenesis

Principal Investigator: Harold F. Blum

Cooperating Unit: Department of Biology, Princeton University
Princeton, New Jersey

Man Years (calendar year 1960):
Total: 1-1/3
Professional: 1
Other: 1/3

Project Description:

I. Quantitative Studies of Carcinogenesis

A. Carcinogenesis by ultraviolet light.

A model that satisfactorily explains the existing data on cancer induction by ultraviolet light in a quantitative sense, was reported in 1958 and 1959. There has been no further direct study in this area during the present year, other than experiments reported below under the heading Studies of the Acute Effects of Ultraviolet Light on Skin. Study of carcinogenesis has been focused this year on a model for chemical carcinogenesis, as reported below.

B. Carcinogenesis by chemical agents.

Continuing the exploration begun last year, a quantitative model for carcinogenesis by chemical agents has been formulated. This model contains the same fundamental assumptions as the one I have already applied to my experimental data on carcinogenesis by ultraviolet light. The equations have, however, a different form, explainable on the basis of temporal relationships of the carcinogenic agent. In the case of ultraviolet light the primary action ceases with the termination of the exposure, with a chemical carcinogen the primary action continues because the agent remains present in the tissue.

Briefly, the model assumes that the rate of proliferation of the cancer cells is proportional to the concentration of the carcinogen in the tissue. A simple exponential die-away of the carcinogen concentration is assumed: Thus:

$$\frac{dV}{Vdt} = src_o(1 - e^{-at}) \qquad (1)$$

Where V is the volume of cells concerned, c_o is the initial concentration of the carcinogen and t is the time elapsed after applying the carcinogen. The constants s, r and a have the following significance; s measures the fraction of the agent which reacts with the tissue, r relates the reaction with the tissue to the rate of cell proliferation, and a describes the time course of disappearance of the carcinogen from the tissue.

By integration of (1) is obtained:

$$\ln V = src_o t + \frac{src_o e^{-at}}{a} + C \qquad (2)$$

For doses of carcinogen repeated at regular intervals one obtains

$$\ln V_d - \ln V_o = src_o t_d + src_o \left[\frac{e^{-ai}}{a} + \frac{e^{-2ai}}{2a} + \cdots \frac{e^{-nai}}{na} \right] - \frac{src_o}{a} \left[1 + \frac{1}{2} + \cdots \frac{1}{n} \right] \qquad (3)$$

where i is the interval between the doses V_o is an initial volume and V_d is a terminal volume at time t_d.

Assuming that V_d and V are constant, as in the model for ultraviolet light, equation (3) may be applied to existing data. Doing so, a first approximation fit is obtained with two sets of data described in the literature for experiments that differ widely in the conditions of dosage, (Crabtree, 1944; Druckrey, 1956) and which seem at first examination to be unreconciliable. The calculation of the partial sums of the series in (3), which is required for a more adequate test of the model would be difficult by ordinary methods because numerous values of a must be tried out. At the moment the problem is being programed for the 650 computer with the cooperation of Professor Roger Pinkham and Mr. Larry Simon, and it is hoped shortly to have a better idea of the applicability of the model to these data.

If the model is found to apply, further calculations will be made using other data to be found in the literature. Depending upon the success in describing existing data it may be advisable to set up some experiments, carefully designed to test the model more definitively.

The model was presented at the Fourth Berkeley (California) Symposium on Statistics and Probability, in July of this year (reference 3).

While it would be premature to claim too great success for the model at the present moment, the first approximation fit is encouraging. Moreover, the basic premises seem altogether reasonable and in accord with the ultraviolet model. In any case the model points up certain things regarding chemical carcinogenesis which seem to have basic importance. The effectiveness of any carcinogen, should depend upon the values of the three constants s, r and a, and under the circumstances it would be difficult to evaluate the "specific" effectiveness of a given carcinogen. For example, a carcinogen might be highly effective because of its ability to react with the tissue, or because of its slow excretion from the tissue. Without an adequate quantitative model we cannot know which.

About the time this model was first formulated, I was asked to participate in a panel on food additives and cancer, before the House Committee on Foreign and Interstate Commerce. This meeting emphasized to me the practical importance of the problem under study here.

If, as indicated in my model the potency of a carcinogen has to be evaluated in terms of two, possibly three functions, then the setting of tolerance levels becomes a very questionable procedure. To do so on the basis of direct tests on laboratory animals, of the kind commonly depended upon by toxicologists, would seem a dangerous procedure - one that could lead to a false sense of security.

Rather than an extensive testing program, the model would suggest well planned studies to get at quantitative relationships and basic mechanisms. This might in the end lead to an appropriate, quantitative evaluation of the carcinogenic potency of types of chemical substances, something that is quite impossible at present.

At the time of the meeting I could only base my arguments on experience with ultraviolet carcinogenesis, which on the bases of my model for that process can be described as cumulative, essentially irreversible, and essentially non-threshold. The model for chemical carcinogenesis also describes a cumulative, essentially irreversible and essentially non-threshold process.

II. Studies of the Acute Effects of Ultraviolet Light on Skin.

This study has a dual objective: (1) to explore the mechanism of the acute hyperplasia that follows at once after dosage with ultraviolet light; and (2) to examine into any relationship that may exist between this process and the cancers that develop only after many doses of ultraviolet light.

My annual report for 1959 described quantitative studies by Dr. Gerald A. Soffen on the effects of single doses of ultraviolet light on the epidermis of the mouse ear. These have been brought to a reasonable point of completion, and Dr. Soffen having been granted his Ph.D. degree has left this laboratory. The results of this study are now being prepared for publication.

It would seem of considerable interest to follow the course of hyperplasia through successive doses of ultraviolet light, since this approaches more nearly the conditions of carcinogenesis by this agent. During the present year a pilot study in this direction was carried out by Mr. Robert Pfeffer working under my supervision. Mouse ears were given two doses of ultraviolet light, spaced one to several days apart. Difficulty was encountered in controlling the anaesthetic for successive doses, this being a principal stumbling block thus far. Several experiments were carried out, but the material is not complete enough to permit more than tentative interpretation and none will be made here.

Because of the construction of a new laboratory building for the Department of Biology at Princeton University, no experimental work was feasible during the summer months. Accordingly I spent part of June, July and August at the Marine Biological Laboratory, Woods Hole, Mass., reading in the library of that institution and writing.

It is hoped that this study may be resumed shortly, in collaboration with Professor Elmer G. Butler, with whom I have worked closely for several years.

III. Studies of the Effect of Non-Ionizing Radiation on Permeability.

No progress has been made in this area during the present year.

IV. Sunlight as an Environmental Factor

The evidence converges to indicate that the sunburning, ultraviolet portion of sunlight is a major factor in cancer of the skin of man. But while the point seems clearly established there remains a good deal of misunderstanding both on the part of the lay public and

of many scientists. This stems largely from lack of understanding both of the spectral character and distribution of sunlight and of the physics and physiology of the skin. During the years I have been working in this field I have felt that I should take every occasion to clarify this situation in writing or speaking on the subject when opportunity arises, and various publications have been directed toward this end (e.g.,references 1 and 2). A number of erroneous ideas still remain current.

The epidemiological aspect of the evidence leaves much to be desired. There is a paucity of measurements supporting the correlation between the distribution of sunlight and the distribution of skin cancer. In no geographical area do we have both appropriate, direct measurements of the carcinogenic radiation, and applicable epidemiological studies. Our only reasonably good correlation, at the moment, is drawn between calculated latitude distribution of the sunburning ultraviolet, based on a relatively few measurements; and the latitude distribution of skin cancer in the United States, culled from the studies of Dorn in 1941. In neither case were the studies made with a correlation between sunlight and skin cancer in view.

Obviously the most careful epidemiological studies may be of little value in the solution of such a problem, unless appropriate measurements of the environmental factor are available. A great difficulty has been the lack of a field instrument to measure and record the carcinogenic radiation, which could be set up in various locations appropriate for epidemiological study. An abortive attempt in this direction was made in 1940-41 from this Institute. Difficulties with instrumentation were encountered, and our entrance into World War II ended the project.

This problem has aroused considerable interest in Australia, where the incidence of skin cancer is high. A group centered at Brisbane is working in this field. They have devised a measuring instrument which has proved promising in field trials. Unfortunately those concerned have found difficulty in obtaining adequate funds for realizing their plans for a correlation of radiation measurements with epidemiological data. The group is fully aware of the difficulties of establishing comparable pathological criteria, as well as comparable radiation measurements, and that both things are essential to a meaningful solution of the problem. One of their principal concerns is the possible role of other environmental factors such as humidity and dust. The geography of Australia seems to lend itself well to such a study.

The Australian group has written me for advice on various occasions, and I have met and talked with some of them when they have visited this country. I am confident that they understand the problems involved, and that they will obtain valuable information if

they can find support.

While there has been some interest in the problem in this country, particularly in our Southwest, there has not been, to the best of my knowledge, any serious survey planned, with the exception of our own attempt mentioned above. Some epidemiological studies are being made, but little or no attention seems to be given to the environmental factor, sunlight. The danger in this situation is that very unsound conclusions regarding etiology, prognosis and prophylactic measures may be drawn from epidemiological studies if the environmental factors concerned are not properly evaluated.

As regards my own position in this area, not being trained either as an instrumental physicist nor as an epidemiologist I do not feel competent to enter actively into either phase of such work. On the other hand, I think I am particularly qualified to help in making liason, or in advising in this area, if the Institute becomes interested. I consider my studies in carcinogenesis of the greater importance, however, at the present time.

Significance to the program of the Cancer Institute: To obtain a better understanding of the mechanism of carcinogenesis, and to explore the implications of any findings as these regard the Public Health.

Proposed course of project: To continue this program.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Blum, Harold F.: Photobiological research with particular reference to skin. J. Am. Med. Assoc. 173: 1353-3157, 1960.

2. Blum, H. F.: Does the melanin pigment of human skin have adaptive value? An essay in human ecology and the evolution of race. Quart. Rev. Biol. (in press)

3. Blum, H. F.: Comparable models for carcinogenesis by ultraviolet light and by chemical agents. In Fourth Berkeley (Calif.) Symposium on Statistics and Probability. Univ. Calif. Press (in press)

4. Blum, H. F.: Complexity and organization. In Form and Strategy in Science (Woodger Festschrift). Cambridge Univ. Press (in press).

Honors and Awards relating to this project:

Honors

Sigma Xi Award for article "Quantitative Aspects of Cancer Induction and Growth: As Illustrated in Carcinogenesis by Ultraviolet Light." in American Scientist 47: 250-260, 1959.

Visiting Scholar at Birmingham Southern College, January 1960.

Seminars and Symposia

Jan. 20-22 Five informal lectures at Birmingham Southern College.

Feb. 11 Lecture "Ultraviolet Light and Cancer", at Philadelphia Skin and Cancer Hospital.

Mar. 21 Discussion of "Can Man be Modified", by Jean Rostand. A talk at "The Symposium", Princeton University.

April 4-6 Participation in Panel on Food Additives before the House Committee on Foreign and Interstate Commerce.

April 27 Lecture, "Vision and the Evolution of Art" at Graduate Forum, Princeton University.

May 11 — Seminar "Vision and the Evolution of Art" at the University of Massachusetts, Amherst, Mass.

July 5 — Seminar paper, "Complexity and Organization", at Marine Biological Laboratory, Woods Hole, Mass.

July 20 — Symposium paper, "Comparable Models for Carcinogenesis by Ultraviolet Light and by Chemical Agents", at Fourth Berkeley Symposium on Statistics and Probability". Berkeley, Calif.

Nov. 15 — Symposium paper, "Genotype, Phenotype and Environment", at Philadelphia Physiological Society.

Serial No. NCI-918
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Research Development Projects in the Field of Electronics

Principal Investigator: Nathan Coffey

Man Years (calendar year 1960):

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To design new electronic equipment as the needs of the Laboratory arise; to modify existing electronic equipment to meet the changes required in an experiment; and to supervise the research development projects in the field of electronics.

Major Findings:

1. Designed and constructed as a single package electron circuitry for a particular electrophoresis device. Investigator's requirements were: 1) low cost, 2) precise control of current, combined with metering system to provide accurately reproducible conditions, 3) to be capable of operating a single electrophoresis cell or two simultaneously. (For Dr. F. Smith.)

2. Designed and constructed analog-to-digital converter and parameter box for insertion of identification and instruction code in computer language, with the combined information stored in punched paper tape. The device was required to cope with the great amount of data being collected from a commercial recording spectrophotometer operated by the investigator. Commercial equipment available for performing these functions offered a number of disadvantages, including:

(a) High cost of basic equipment plus engineering time required to adapt to particular application.

(b) Necessity of making a number of modifications to the spectrophotometer to make it compatible with existing analog-to-digital equipment.

(c) Probability that commercial equipment adapted for this use would require major modifications if, at some later date, it became desirable to use the equipment for data collection from some other source.

Design of the present equipment was undertaken with the following stipulations:

(a) Cost to be held to the lowest figure compatible with required accuracy and reliability.

(b) To facilitate approximate duplication by others, low-cost, non-computer-type electronic devices normally available on an off-the-shelf basis (for example, decade scalers, test equipment) were incorporated as sub-assemblies to hold actual construction and wiring to a minimum.

(c) Modification of the spectrophotometer was to be limited to the simple attachment of a transducer to provide information to the analog-to-digital converter.

(d) Design was to be such that transfer to another type of data-collecting device could be made simply.

Conception, design, construction, and testing have required the better part of a year. The device is now attached to a spectrophotometer and operation of the over-all system is being evaluated. Total cost of components, including tape punch, was approximately $1,700. (For Dr. J. Reid.)

3. Routine maintenance of all electronic equipment in the Laboratory has additionally been carried on.

Significance to the program of the Cancer Institute: Provides capability of securing refined procedures for sensing and measuring physiological factors, and for logging pertinent data in a form most suitable for manipulation.

Proposed course of project: To continue to provide a facility for combining advanced electronic techniques with advanced procedures in physiological research to insure rapid and accurate collection of experimental data.

Part B included No

Serial No. NCI-930 (formerly NCI-691, Radiation Branch)

1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Repair of X-ray Damage in Cell Lines of the Chinese Hamster Grown in Culture

Principal Investigator: Mortimer M. Elkind

Man Years (calendar year 1960):
Total: None - Completed project
Professional:
Other:

Project Description: No new information since 1959 Report. Project completed except for additional Part B material reported here.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Elkind, M. M.: Cellular aspects of tumor therapy. Radiology 74: 529-541, 1960.

Alper, T., Gillies, N. E., and Elkind, M. M.: The sigmoid survival curve in radiobiology. Nature 186: 1062-1063, 1960.

Elkind, M. M., and Sutton, H.: Radiation effects on mammalian cells grown in culture. I. Repair of x-ray damage in surviving, Chinese hamster cells. Radiation Res. 13: 556-593, 1960.

Honors and Awards relating to this project:

Title: "Time-dose factors in the irradiation of mammalian cells in vitro"

Place: National Cancer Institute, Radiation Study Section's invited conference on experimental advances in radiobiology; Carmel, California, May 5, 1960.

Title: "The implications of cellular recovery to tumor therapy"

Place: Radiology Department, University of California Medical School, San Francisco, California; May 12, 1960.

Title: "Repair of sublethal damage in mammalian cells grown in culture"

Place: Experimental Radiopathology Research Unit, Medical Research Council, Hammersmith Hospital, London, England; September 2, 1960.

Serial No. NCI-930(a) (formerly
NCI-691(a) Radiation Branch)
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The Dependence of Lethality on X-ray Dose Rate in Chinese Hamster Cells Grown in Culture

Principal Investigator: Mortimer M. Elkind

Man Years (calendar year 1960):
Total:) 1-2/3
Professional: 1/3
Other: 1-1/3

Project Description:

Objectives: The observation that surviving cells to x-irradiation rapidly repair their damage without a time lag (See 1959 Project No. NCI-691) suggests that lethality should be dose rate dependent. Specifically, this observation suggests that at reduced dose rates the killing effect (suppression of unlimited division) should be reduced.

Methods Employed: Improved single cell tissue culture techniques were used for purposes of: 1) making dense cell suspensions; 2) irradiating under controlled conditions; and 3) assaying for surviving cells. Fifty-five Kv x-rays were used.

Major Findings: This work is in progress. Thus far we have found that reduced dose rates have a greater rather than lesser killing effect on Chinese hamster cells grown in cultures as indicated by the mean lethal dose. The dose rate range from 720 rad min. to 9 rad min. is under investigation. The data suggests that the latter dose rate decreases the mean lethal dose by about a factor of 2 compared to the former. However, although the mean lethal dose decreases with decreasing dose rate, the hitness number increases. These findings are preliminary since completely adequate controls have not as yet been worked out.

Significance to Cancer Research: The apparent anomolous nature of these findings are of significance primarily as they relate to the information derived from the 1959 Project No. NCI-691.

Proposed Course of Project: This work will be pursued in order to seek an explanation of these seemingly anomolous results. Due to practical limitations (equipment, personnel, and to some extent techniques) the range of dose rates which can be examined with good experimental control is limited. The importance of understanding these results justifies considerable additional effort.

Part B included No

Serial No. NCI-930(b) (formerly NCI-691(b)
Radiation Branch)
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The Dependence of X-Ray Survival on Cellular Multiplicity

Principal Investigator: Mortimer M. Elkind

Man Years (calendar year 1960):
Total: 3-1/3
Professional: 1/3
Other: 3

Project Description:

Objectives: Pursuing the work reported in 1959, a seemingly anomalous situation was observed in the growth versus survival kinetics in recovered, surviving Chinese hamster cells. The anomaly consisted of an inconsistency between the postirradiation growth as opposed to survivability of cells which had survived an initial exposure. The observation was made particularly apparent when experiments were performed with log phase cells. The possibility was recognized that if the implications of these initial findings were borne out by additional experiments, then one of the basic tenets of radiobiology would become questionable. That is, it has come to be assumed in radiobiology that the site structure in cells lethally affected by ionizing radiation is discrete. One of the minimal requirements of this assumption is that a cell in a group of cells should survive as an individual and uninfluenced by the proximity of its neighbors. The anomalous results referred to above suggest that under certain conditions, cells in a group do not survive as individuals, but rather in a way that indicates that two or more cells can be killed under conditions which might otherwise kill only one cell. The objective of this project, therefore, has been to investigate this possibility vis-a-vis the anomaly referred to.

Methods Employed: See Project No. NCI-930(a).

Major Findings: Thus far, our studies have dealt with a detailed examination of the course of events following a single exposure of 433 rads (which corresponds to about 20% survival for log phase cells) and the period starting from 20 hours on postirradiation. We have found that: 1) Survivors suffer a delay in growth of 7 - 8 hours after which they grow

as rapidly as unirradiated cells, (i.e. doubling time 11 hours). 2) By at least 20 hours, and probably even sooner, surviving cells have completely repaired their sublethal damage and they and their progeny survive in a way consistent with no interactions between them. 3) From about 20 to 35 hours, surviving cells and their progeny interact such that the probability of killing each cell in a potential colony forming unit decreases. This latter observation is true only if the surviving cells and their progeny are not separated prior to exposure. Thus it appears that after recovery, a surviving cell subjected to additional exposure can transfer damage to a neighboring cell.

Significance to the National Cancer Institute: Radiation can induce malignancy or kill malignant cells. It is self evident, therefore, that a basic study such as this is quite important to cancer research.

Proposed Course of the Project: These findings will be extended to learn as much as one can about the nature of the cell-to-cell interaction related to radiation lethality. Of concern, is an almost axiomatic principle in radiobiology which may have to be modified if not discarded if the interpretation of our present results is sustained by future experiments.

Part B included No

Serial No. NCI-930(d) [formerly NCI-691(d), Radiation Branch]

1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: The Influence of Drugs on Lethality and Recovery in X-Irradiated Mammalian Cells Grown in Culture

Principal Investigator: Mortimer M. Elkind, Ph. D.

Other Investigator: William C. Mohler, M. D. *

Cooperating Unit: *Clinical Pharmacology and Experimental Therapeutics Section, General Medicine Branch, National Cancer Institute

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: The influence of drugs on the x-ray response of mammalian cells is of importance for theoretical and applied reasons. The effect of a drug can be expected to be found on the "killing" of cells by x-rays and also on the recovery of cells which survive exposure. Recently, halogenated pyrimidines have become of interest. In this study the action of 5-bromo deoxyuridine (BUDR) is being examined in a Chinese hamster cell line which is being used in related work [Project Serial No. NCI-930(a)]. As time and conditions permit, the influence of other drugs is being examined.

Methods Employed: See Project No. NCI-930(a).

Major Findings: The results with BUDR indicate that: 1) BUDR can increase the x-ray sensitivity of this cell line. 2) This increase is accompanied by alterations in both of the major survival parameters (mean lethal doses (MLD), and extrapolation number). 3) The effects on MLD and extrapolation number may be separable under some circumstances. 4) The pattern

of recovery of surviving cells is altered by BUDR. 5) The alteration of x-ray sensitivity by BUDR is not merely the result of non-specific cell injury since other drugs (8-azoguanine and amino ethyl isothiuronium) alter one x-ray survival parameter (MLD) without affecting the other.

Significance to Cancer Research: The action of drugs and chemicals may be of help in the identification of the sites of action of lethal irradiation and understanding the radiation response of malignant tissues. The ability of drugs to alter the initial radiosensitivity of cells and to alter the recovery of cells surviving irradiation may have importance in clinical radiotherapy.

Proposed Course of Project: The effects of BUDR will be investigated in greater detail in an effort to correlate the changes in x-ray response with the degree of incorporation of BUDR into desoxyribonucleic acid. The effects of other drugs will be investigated when and if practicable.

Part B included No

Serial No. NCI-900
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Studies in Tumor-Host Relationships

Principal Investigators: Julius White, Florence Kate Millar and Jane Nicolet Toal

Man Years (calendar year 1960):
Total: 6
Professional: 2
Other: 4

Project Description:

Objectives:

1) To define and study changes in body composition and physiology which occur in tumor-bearing rats and to discover how the presence of a tumor accomplishes these changes. Present emphasis is directed to the sodium retention and adrenal hypertrophy previously reported in Walker tumor-bearing rats.

2) To continue the study of growth-stimulating factor(s) present in tumor tissue.

Methods Employed: The metabolism of tumor-bearing rats was investigated by observing the intake and excretion of electrolytes and nitrogen followed by carcass and tumor analysis. Distribution of electrolytes and nitrogen in carcasses and tumors of the animals was calculated from the balance data and the analytical results.

A major portion of the investigations was carried out on Sprague-Dawley rats bearing the Walker Carcinosarcoma 256 subcutaneously. To provide information on another type of tumor, Wistar rats bearing the Murphy-Sturm lymphosarcoma intramuscularly were used for some experiments. The 1959 Annual Report described the diets employed and the procedures for metabolic balance studies of electrolytes (Na, K, Cl) and N and for subsequent carcass

and tumor analyses (water, fat, N, Na, K, and Cl). Electrolytes were also determined in serum and in samples of skin, muscle and bone in a few experiments. During the past year the above procedures have been used to study the following:

Both subcutaneous and intramuscular transplant sites were studied for the Walker tumor and for the Murphy-Sturm tumor.

The influence of age on carcass and tumor composition was studied in Walker tumor-bearing rats. Tumor transplantation into 90-gram rats and into 180-gram rats was compared after 26 and 36 days, respectively.

For the purpose of evaluating the effect of a decreased food intake which frequently occurs in tumor-bearing rats, normal Sprague-Dawley rats were maintained on a restricted food intake (5 gm. of a 20% casein diet per day for 5 weeks or 3 gm. per day for 4 weeks).

The investigation of adrenal hypertrophy and histologic change in rats bearing the Walker tumor subcutaneously was continued. Tumor-bearing rats were sacrificed at different times after tumor transplantation and adrenal weights and tumor size (percent of body weight) were compared. The rats ingested either a 20% casein diet (C-20) or the same diet with an enhanced level of NaCl (C-20 + 0.9% NaCl).

Major Findings:

Na retention and distribution:

As reported in 1959, rats bearing the Walker tumor subcutaneously and remaining in nitrogen balance showed pronounced retention of Na beginning 15-25 days after tumor transplantation (when the tumor was 10% or more of the body weight) and lasting throughout the ensuing period of tumor growth. Analyses of carcasses and tumors of these animals showed that the amount of Na found in the tumor accounted for almost all of the total Na retained during the study.

Rats bearing the Murphy-Sturm lymphosarcoma also showed pronounced Na retention beginning about 7-10 days after tumor transplantation, lasting 3 to 7 days, and returning to normal or less than normal levels for the remainder of the period studied. Thus the total Na retained by the Murphy-Sturm tumor-bearing rats was much less than that retained by the Walker tumor-bearing rats.

Carcass Composition:

When the concentrations of nitrogen and electrolytes in carcasses of rats bearing the Walker tumor subcutaneously were expressed on a fat-free wet weight basis, it was found that Na, Cl and N were increased above normal values and K and H_2O decreased. Na concentration in the carcass increased as tumors became larger and was directly related to tumor size when tumors were at least 20% of body weight. Serum Na, K and Cl concentrations of other comparable rats were within the normal range. Rats transplanted at 90 gm. with the Walker tumor showed similar increased carcass Na and Cl concentrations and decreased K.

Attempts to locate the site of the abnormal Na and Cl carcass concentration showed there was some elevation of these ion concentrations in samples of skin from tumor-bearing rats but not in samples of muscle and bone. These increases in concentration of Na and Cl in the skin were not enough to account for the increases found in total carcass.

Carcasses of Wistar rats bearing the Murphy-Sturm lymphosarcoma intramuscularly showed changes similar to the Walker tumor-bearing rats.

There are reports in the literature of similar changes in carcass composition for undernourished normal rats (Na, Cl and N increased; K and H_2O decreased). In our studies, normal (non-tumor-bearing) rats on a restricted food intake of 5 grams per day for 5 weeks remained in nitrogen balance and had normal carcass concentrations. Normal rats ingesting 3 grams a day for 4 weeks did not remain in nitrogen balance and the carcass concentrations of Na increased, Cl slightly increased and K remained normal. With respect to carcass composition, the tumor-bearing rat in positive nitrogen balance resembles more closely a non-tumor-bearing rat in negative nitrogen balance. However, the positive nitrogen balance of the tumor-bearing rat (carcass plus tumor) does not measure the nitrogen balance of the carcass alone.

Tumor Composition:

A comparison of the Walker tumor 15 days after transplantation with tumors 25-30 days old showed that the tumor composition was altered in relation to the amount and degree of necrosis present. Necrotic tissue had higher concentrations of Na and Cl and lower concentrations of K and N than viable tissue. These were the directions of the concentration changes found in whole tumor with increasing size, with the exception that there was no alteration in nitrogen concentration. The Na/K ratio of whole Walker tumor ranged from 1 to 3.

The Na/K ratio of the non-necrotic Murphy-Sturm lymphosarcoma ranged from 0.5 to 0.8. This tumor, grown intramuscularly, showed a K concentration similar to that of muscle but a Na concentration about double that of muscle.

Effect of tumor site upon Na retention:

As mentioned earlier, rather different patterns of Na retention were exhibited by rats bearing the Murphy-Sturm lymphosarcoma intramuscularly in the leg as compared to rats bearing the Walker tumor subcutaneously in the back. Since the difference might have been related to the tumor per se, the site of transplantation, or to both, the Walker tumor was studied in the intramuscular (leg) site and the Murphy-Sturm tumor in the subcutaneous site. The Na excretion-retention pattern as well as tumor size was similar for the rats bearing the Murphy-Sturm tumor at either site at 15-17 days.

For rats bearing the Walker tumor intramuscularly in the leg, the tumor sizes were not as large as frequently obtained subcutaneously at 28 days. Therefore, the drop in Na excretion was not as severe for the intramuscularly transplanted rats, although the Na excretion-retention patterns were roughly similar in the two sites.

Adrenal hypertrophy:

The adrenal weights of rats bearing the Walker tumor subcutaneously and ingesting a diet with normal Na level (C-20) exceeded the normal range when the tumors reached about 20% of body weight. The adrenals continued to increase with time and with increased tumor size. Enlarged sinusoids were observed in the adrenals of rats which had tumors at least 20% of body weight.

When increased adrenal weight alone is taken as an indication of adrenal hypertrophy, no distinction can be made between enlargement resulting from increased sinusoidal spaces, increased width of the zona glomerulosa as a result of an increased need to retain Na, or other causes. Further histological studies are necessary to distinguish these effects and their relation to salt intake and tumor size.

Significance to the program of the Cancer Institute: The various effects which have been described above in tumor-bearing rats (adrenal changes, alterations in content and distribution of the major electrolytes of the body, changes in carcass composition) have been the results, directly or indirectly, of the presence of the tumor. Information on the manner and means by which a

growing tumor distorts the normal physiology of the host is pertinent to the development of therapy.

Proposed course of project:

a) Effects of a growing tumor on the electrolyte physiology of the host will be continued as follows: (1) Studies of adrenal changes will continue; special attention will be given to a more detailed evaluation of histological changes. (2) After appropriate methods have been selected or devised, aldosterone levels in tumor-bearing rats will be investigated to further understanding of the Na retaining mechanism.

b) Studies on the effect of a tumor on the nitrogen metabolism of the host will be continued.

c) Studies of the growth-stimulating properties of tumor tissue will be continued in an attempt to isolate any factor(s) of interest other than the Na ion. Particular attention will be paid to the separation of protein from nucleic acid moities. The efficiency of tumor tissue as a source of nitrogen for supporting growth of normal and tumor-bearing animals will continue.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Toal, J. N., Millar, F. K., Brooks, R. H., and White, J.: Sodium retention by rats bearing the Walker Carcinosarcoma 256. (Accepted for publication; to appear in Am. J. Physiol., Jan. 1961.)

Serial No. NCI-905
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Iodide-Concentrating Mechanism of the Thyroid Gland

Principal Investigator: Seymour H. Wollman

Other Investigator: Rosalind Pitt-Rivers, Visiting Scientist, Laboratory of Clinical Endocrinology, NIAMD (Project Serial Number not available)

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: To learn about (a) the mechanism by which the thyroid gland maintains a concentration of iodide elevated above that in the blood, and (b) the mechanism by which the thyroid gland accumulates protein-bound iodine.

Major Findings: This year investigation has been restricted to examination of blood from veins draining the thyroid gland of the rat. In one type of study the thyroid gland was labeled with radioiodine and from comparison of arterial and venous blood it was possible to determine the compounds the thyroid gland was secreting. Results as yet are only preliminary and will be reported on next year.

Significance to the program of the Cancer Institute: This work is leading to an understanding of the relative importance of various factors in controlling the accumulation of radioisotopes by tissues.

Proposed course of project: This project will be continued with somewhat more emphasis on investigation of the role of blood flow to the thyroid in the accumulation of radioiodine by the thyroid gland.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Wollman, S. H.: Concentration and organic binding of radioiodide by the thyroid gland. Annals of the N. Y. Acad. Science 86: 354-361, 1960.

Honors and Awards relating to this project:

Invited lectures:

1. Concentration of iodide by the thyroid gland. Presented at the Gordon Research Conference, "Cell Structure and Function," June 20-24, 1960.

2. Synthesis and secretion of thyroid hormones. Presented at the Combined Clinic, College of Physicians and Surgeons, Columbia University, November 5, 1960.

Serial No. NCI-906
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Properties of Transplantable Thyroid Tumors

Principal Investigator: Seymour H. Wollman

Other Investigators:
Dr. L. Warren, LPT, NIAMD
Dr. S. S. Spicer, LPH, NIAMD
Dr. J. H. Tjio, LPH, NIAMD
Dr. M. Rechcigl, LB, NCI

Cooperating Units: NIAMD, Laboratory of Pharmacology and Toxicology - Dr. Leonard Warren (Project Serial No. not available)

NIAMD, Laboratory of Pathology and Histochemistry - Drs. S. S. Spicer and J. H. Tjio (Project Serial No. not available)

NCI, Laboratory of Biochemistry - Dr. J. Rechcigl (Project Serial No. NCI-320)

Related Projects: Clinical Endocrinology Branch, NIAMD.

Radiation Branch, NCI - esp. Dr. F. Smith and Dr. Gordon Sharp.

Man Years (calendar year 1960):

Total:	1-1/3
Professional:	1/3
Other:	1

Project Description:

Objectives: To study properties of transplantable thyroid tumors in the rat: Variety of histologic types and their progression, correlation of histology with biochemistry and physiology of the tumors.

Major Findings:

1. Tumors of line 1-5A can maintain a concentration of radioiodide elevated above that of blood although these tumors cannot form appreciable protein-bound I^{131}. Although the ability to concentrate iodide is appreciable, it is less than in normal thyroid glands. The reason for the impairment of the ability to concentrate radioiodide was investigated. The impaired ability seems to be a consequence of a gross impairment in transport of radioiodide from blood to tumor. In addition, however, there is also some impairment in transport from tissue to blood as well.

2. Dr. J. H. Tjio has examined the chromosome complement of three lines of tumors and found that two anaplastic lines had deletions of 4 and 7 chromosomes, respectively, whereas a functional line had a deletion of only 1 chromosome.

3. Dr. M. Rechcigl has studied the effect of rapidly growing anaplastic tumors of line 1-3 on the concentration of catalase in the rat liver. He has found that when tumors were present the liver catalase level was decreased in normal and in hypophysectomized rats, but that in hypophysectomized rats without tumors the liver catalase level is relatively normal. This appears to exclude the pituitary gland from a major role in controlling the drop in liver catalase in rats bearing tumors.

Significance to the program of the Cancer Institute: These tumors are valuable because of the variety of their easily identified histologic and biochemical characteristics. Physiologic studies will give insights into the correlation of structure and function.

Proposed course of project: Studies of the physiology of these tumors will be continued.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Spicer, S. S., Wollman, S. H., and Warren, L.: Histochemical demonstration of sialomucin in transplantable thyroid carcinoma. Am. J. Pathology 37: 599-611, 1960.

Wollman, S. H., and Reed, F. E.: Radioiodide transport in an iodide-concentrating tumor of the thyroid gland. Am. J. Physiology 199: 417-418, 1960.

Wollman, S. H.: Effects of feeding thiouracil on the thyroid glands of rats. J. Nat. Cancer Inst. (in press).

Serial No. NCI-919
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Investigation of Urinary Excretion of Nucleic Acid Congeners in Leukemia

Principal Investigator: James C. Reid

Other Investigator: William R. Bell (Post-Sophomore Fellow)

Cooperating Units: General Medicine Branch, NCI,
Project Serial No. NCI-4-719 (c)

Man Years (calendar year 1960)

Total:	3
Professional:	1
Other:	2

Project Description:

Objectives: The purpose of this project is to study the excretion pattern of nucleic acid congeners and other substances employing methods developed in this Laboratory.

Methods Employed: The methods used are primarily chromatographic. Machine methods of data processing are being introduced.

Major Findings:

Certain residual technical difficulties in the chromatographic procedure, mentioned in our last report, have been resolved satisfactorily and the system is now running on a routine basis.

Data are available on a group of eight urine specimens which comprises two normals on an unrestricted diet, one of these normals on the low-purine, low-ribose control diet to which all our patients are confined, two cases of untreated and acute myeloblastic leukemia, two cases of untreated acute

lymphoblastic leukemia and a specimen from one of these lymphoblastic cases after a course of combined drug therapy which produced remission.

Effect of Diet:

Dietary composition exerts an important effect on the normal urinary pattern. A freely selected diet elevates many of the peaks of the control pattern and introduces some peaks not seen in the control. This is seen in all fractions although the nucleoside fraction appears least affected. A number of peaks are not affected and these should prove useful as metabolic reference points. An easily recognizable similarity exists between the normal patterns, which offers an indirect indication that the chromatographic system is functioning reproducibly.

Myeloblastic Leukemia:

The two myeloblastic specimens also exhibit a similar pattern. Although there are quantitative variations in some regions, qualitatively these patterns exhibit distinct differences from the normal control. There is an extra peak in the main free base fraction, an extra peak in the middle of the uracil-thymine-thymidine fraction, an extra peak toward the end of the nucleoside fraction and apparent loss of part of a poorly characterized peak in the nucleotide fraction. The uridine fraction exhibits a change which suggests loss of a normal peak and appearance of a new one although it is possible that two closely spaced peaks of the normal pattern are merged.

It should be mentioned that the terms applied above, such as "nucleoside fraction," are nominal designations based on the position of known compounds run through the system and are not based as yet on chemical examinations. In fact, it is known from preliminary work that all of these "peaks" are complex mixtures.

Lymphoblastic Leukemia:

The lymphoblastic pattern also differs distinctly from the normal. It closely resembles the myeloblastic pattern in the free base fraction, the uridine fraction and the uracil-thymine-thymidine fraction although there is a suggestion of some reduction in the components of the latter. There is additional structure toward the end of the nucleoside fraction however, relative to the myeloblastic pattern and the nucleotide fraction exhibits an alteration which appears to consist of a loss of material from the late region and the appearance of a new entry in the earlier part. There is a marked reduction

of the total nucleotide fraction. Thus there is a suggestion that the profiles may be capable of differentiating the two types of disease.

Remission:

Data from the treated lymphoblastic patient in remission has not been fully evaluated but there is a clear-cut increase in total material in the nucleotide fraction and a return toward its normal pattern. This trend, if substantiated, would suggest that the pattern changes are in fact correlated with the leukemic state and moreover with the clinical status of the patient.

Quantitation:

It must be emphasized that the comments above are highly tentative and that a considerably larger number of specimens must be examined before the significance of the observations can be evaluated. Beyond this lies the crucial problem of estimating the amounts of the individual components of the peaks. Without this information it is impossible to judge, except in the most exaggerated cases, whether peaks are dropping out or being merged into adjacent peaks, whether more peaks are appearing or old ones are shifting position or being more highly resolved and whether highly significant fluctuations of an individual component of a mixed peak fail to influence the total significantly and are therefore missed. In addition, when patterns differ even the roughest sort of quantitative comparisons are hazardous because of uncertainty as to where to establish the break-points.

As was stated in the last report, this problem of quantitation is being approached in collaboration with Dr. Pratt, Laboratory of Physiology, by means of vector analysis of the ultraviolet mixture spectra, a technique which requires the aid of a digital computer (see Project Serial No. NCI-929). We have not succeeded in examining any urinary data however, because of delays in procurement of digitizing gear and difficulties experienced in attempting to use the central computing facility for a problem of this type. The latter difficulties will not be detailed here but it may be mentioned that they stem from: 1) the highly varied character of the preliminary computations and the impossibility of foreseeing all the technical problems which emerge from the chemical operations; 2) the need for speedy computation since each step depends upon the result of the preceding computation; 3) the need for rapid programming of limited individual operations as necessity arises; and 4) the impossibility of the chemist communicating with the programmer with the precision necessary to write the detailed machine instructions and of the chemist communicating with the console operator in sufficient detail for the latter to select

from a library with which he is unfamiliar, the proper programs to use with a given set of data.

Our experience in this area has led us to the conclusion that a small digital computer is necessary in the Laboratory in order to permit the chemist to carry out those phases of the computation which cannot be done without a detailed grasp of the laboratory factors. With such a "satellite" machine he can carry the computation to the point where it becomes a standardized operation such as matrix inversion which can then be sent to the larger machine with communication problems reduced to manageable proportions, and where the greater speed and capacity of the larger machine can be effectively utilized. It is entirely feasible to do in the laboratory the sort of programming needed for the simpler satellite machine. Indeed if the digital computer is to achieve any real utility for the laboratory it will have to be reduced to the status of any other research device such as the spectrophotometer itself.

Significance to the program of the Cancer Institute: This study is an attempt to uncover biochemical peculiarities of the leukemic host which will be valuable

1) To increase knowledge of the nature of the disease.
2) To aid in diagnosis and evaluation of clinical status.
3) To increase knowledge of the mechanism of action of drugs.

Proposed course of project:

1) More leukemia patients will be studied.

2) Development effort will be concentrated on the problem of the analysis of the composition of the peaks, particularly those which differ from the normal pattern.

Part B included No

Serial No. NCI-920
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Investigation of Compound A-20

Principal Investigator: James C. Reid

Man Years (calendar year 1960):
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objectives: Identification of what is believed to be a new compound found in rat liver.

Methods Employed: Chromatographic methods are used for isolation of the substance. The methods of physical and organic chemistry are used to accumulate information which will permit an identification.

Major Findings: Several schemes have been tested in an attempt to develop both a synthetic procedure for the compound and degradative procedures for converting it to identifiable fragments. These efforts have been unsuccessful.

Significance to the program of the Cancer Institute: The substance studied is thought to be related to protein metabolism which is an important feature of tumor growth.

Proposed course of project: Work on this project has been suspended temporarily in order to concentrate on more fruitful activities.

Part B included No

Serial No. NCI-925
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Protein Synthesis and Its Antagonism in Normal and Tumor Cells

Principal Investigator: Marco Rabinovitz

Man Years (calendar year 1960):

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

a) To study, in both normal and tumor cells, the route or routes taken by amino acids in the formation of protein, through an analysis of the biochemical lesions introduced within the pathway by specific inhibitors of protein synthesis.

b) Where applicable, to extend the information obtained in the first objective to the tumor-bearing animal with the view of selective inhibition of protein synthesis in cancer cells.

Methods Employed:

a) Organic synthetic techniques are developed for preparation of potentially effective amino acid antagonists.

b) Incorporation of carbon-14 or tritium labeled amino acids into protein of isolated cells is measured. Such studies involve radioactive counting techniques and stripping film radioautography. Respiration and anaerobic glycolysis are followed by standard manometric techniques.

c) Subcellular components are isolated by differential centrifugation. These components are further fractionated by extraction, hydrolysis or chromatographic techniques. The characteristics of the block in amino acid utilization within these fractions are evaluated.

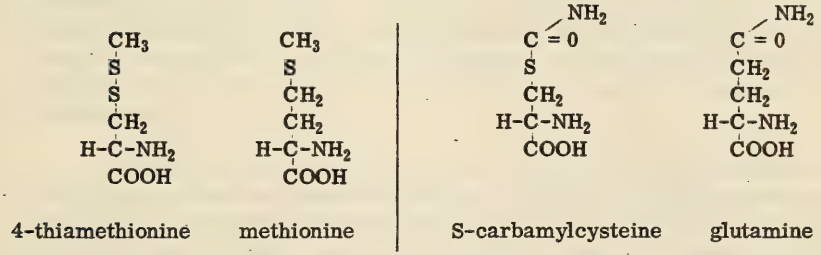
d) Tumor-bearing animals are placed on appropriate dietary regimens and the effect of amino acid antagonists on toxicity, tumor growth and protein synthesis are determined.

Major Findings:

I. Attempts to introduce permanent lesions within the protein synthetic pathway.

(a) Amino acid analogues with highly reactive functional groups

Studies during the last few years in many laboratories have indicated that most amino acid antagonists are incorporated into completed protein molecules in place of their corresponding metabolite. Only one case of a block in the protein synthetic pathway has been reported (J. Biol. Chem. 234: 2091, 1959), and this block was both prevented and reversed by the corresponding metabolite. In an attempt to introduce a more permanent lesion, two amino acid analogues with highly reactive functional groups were synthesized in the hope that they may interact at specific sites in the intermediate stages of protein synthesis. The formulae of these analogues, together with their corresponding metabolites, are shown below:



4-thiamethionine | methionine | S-carbamylcysteine | glutamine

4-Thiamethionine was a potent inhibitor of the incorporation of labeled methionine, leucine, valine, lysine and phenylalanine into protein of Ehrlich ascites tumor cells in vitro. The inhibition was not prevented by methionine. Glutathione prevented the inhibition by reacting with the analogue. This could be followed by paper chromatography of the reaction mixture. However, after the inhibition had been initiated it could not be reversed by glutathione.

Glucose both prevented and reversed the inhibition, thus indicating that 4-thiamethionine interfered with the oxidative energy supply supporting protein synthesis.

S-Carbamyl cysteine inhibited the incorporation of glutamine-C^{14} as well as other amino acids into protein of Ehrlich ascites tumor cells. As in the case of 4-thiamethionine, this inhibition could not be prevented by the corresponding metabolite, glutamine. A striking characteristic of its inhibition of glutamine incorporation, was the fact that the percent inhibition was greater in the presence of higher concentrations of glutamine. This was shown to be due to its interference with the general stimulation of amino acid incorporation by glutamine (J. Biol. Chem. 222: 879, 1956), in addition to its inhibition of incorporation. The results indicate that the stimulation of amino acid incorporation seen in the presence of added glutamine may not be due to the role of glutamine as a limiting amino acid, but involves its prior metabolism.

(b) Amino acid antagonist mixtures

Since individual amino acid antagonists have been shown to compete with their corresponding metabolite for incorporation into completed protein molecules, it was felt that mixtures of analogues may produce a block in the synthetic pathway by producing intermediates with multiple unnatural residues. Two potent antagonists of their corresponding metabolites, α-amino-β-chlorobutyric acid (valine analogue) and S-(β-aminoethyl) cysteine (lysine analogue) were studied alone and together in the presence of their labeled metabolites and other labeled amino acids. As a mixture they partially inhibited the incorporation into protein of several amino acids other than their metabolites. However, the inhibition was not completely prevented by the presence of their metabolites. In general, the results suggest that a large number of unnatural residues may be tolerated by the protein synthetic mechanism.

Another type of mixture, one of amino acids antagonists which individually block protein synthesis was studied. Methionine sulfoximine interferes with the incorporation of several amino acids into Ehrlich ascites cell protein by preventing the biosynthesis of the essential amino acid, glutamine. Only 50 percent inhibition can be obtained (J. Biol. Chem. 222: 879, 1956). O-Methylthreonine will partially inhibit protein synthesis by preventing utilization of isoleucine (J. Am. Chem. Soc. 77: 3109, 1955). These inhibitions are prevented by their corresponding metabolites. When the inhibitors are present together, the degree of inhibition is not additive and not greater than that found in the presence of methionine sulfoximine alone.

One plausible interpretation of the above findings could exist at the cellular level. Some cells may have a high concentration of the amino acid metabolites and thus should be resistant to both antagonists, while others, completely inhibited by one, would show no further effect in the presence of both. To test this possibility, cells were incubated with tritium labeled leucine, in the presence of each inhibitor, both singly and together, and in the absence and presence of the corresponding metabolites. Smears were prepared and were subjected to stripping film radioautography. Grain counts of individual cells supported the observations with total protein preparations. However, two classes of cells were not observed. In the presence of both inhibitors the variability in grain counts per cell was actually much lower than for either inhibitor alone. The results suggest that resistance to the action of the inhibitors is not due to variations among cells but is a metabolic characteristic common to all.

II. Inhibition of protein synthesis in Ehrlich ascites tumor cells by puromycin.

Puromycin, an antibiotic which has been found of interest in cancer chemotherapy, is a potent inhibitor of amino acid incorporation. The following characteristics of its behavior at the sub-cellular level indicate that this material interrupts the protein synthetic pathway in a unique manner:

1. At lower temperatures, puromycin can markedly decrease incorporation into the ribosomal protein but not inhibit the incorporation into soluble protein.

2. Puromycin eliminates the lag phase in the labeling of soluble protein at 22° C. This results in an initial two-fold stimulation of labeling of the soluble protein while the labeling of the ribosomal protein lowered to one-third.

3. The antibiotic causes a precipitous drop in the specific radioactivity of ribosomal protein when it is added to cells in which labeling of this fraction had reached a steady state.

These results appear to be compatible with the postulation that puromycin inhibits protein synthesis at a rate limiting step of the ribosomal synthetic assembly, and, that under appropriate conditions, poorly ordered protein molecules are discharged when this block occurs.

III. Inhibition analysis of the participation of soluble ribonucleic acid in protein synthesis.

Recent studies with broken cell preparations have supported the view that amino acids bound to soluble ribonucleic acid of the cytoplasm (S-RNA) are transferred to ribosomal protein. Accordingly, in intact cells, amino acid antagonists should interfere with the labeling of both S-RNA and protein by its metabolite. This has been found to hold with several labeled amino acids and their antimetabolites, but one case was found where the analogue blocked protein labeling without affecting uptake by S-RNA. This case is currently being investigated.

Significance to the Program of the Cancer Institute: These studies utilize the action of amino acid antimetabolites as a means of comparing protein anabolism in normal and cancer cells. In addition, structure-activity relationship for producing antagonism and studies on mechanism of inhibition, both in vitro and in vivo, should furnish basic data of potential use for selective inhibition of cell types and specific protein-forming systems.

Proposed Course of Project: It is hoped to carry on, to some extent, all aspects of the program. The most interesting developments arising during the course of investigation will be extended at the expense of others.

Part B included No

Serial No. NCI-927
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Sialic Acid in the Thyroid Gland

Principal Investigators: Seymour H. Wollman
Leonard Warren, NIAMD

Cooperating Unit: NIAMD, Laboratory of Pharmacology and Toxicology, Dr. Leonard Warren (Project Serial Number not available)

Related Project: Dr. Robert W. Bates, Section of Endocrinology, Laboratory of Nutrition and Endocrinology, NIAMD

Man Years (calendar year 1960):

Total:	2/3
Professional:	1/3
Other:	1/3

Project Description:

Objectives: To study how the concentration of sialic acid in the thyroid gland varies with physiologic state of the gland.

Major Findings: Confirmation of data reported previously.

Significance to the program of the Cancer Institute: Sialic acid is a prominent component of some transplantable thyroid tumors. Studies on sialic acid in the normal gland were desirable to get insights into reasons for the presence of the sialic acid in tumors.

Proposed course of project: This project has been completed.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Wollman, S. H., and Warren, L.: Effects of thyrotropin and thiouracil on the sialic acid concentration in the thyroid gland. Biochim. Biophys. Acta (in press).

Serial No. NCI-928
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Intracellular Colloid Droplets

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. S. S. Spicer, NIAMD

Cooperating Unit: Section of Biophysical Histology, NIAMD
(Project Serial Number not available)

Man Years (calendar year 1960):
Total: 1/3
Professional: 0
Other: 1/3

Project Description:

Objectives: To study the properties of intracellular colloid droplets in the thyroid gland in order to decide whether they are newly formed colloid or colloid which has been resorbed from the lumen of the follicle.

Major Findings: We have attacked this problem using high resolution autoradiography and histochemical techniques. The studies are now in progress and will be reported on fully in the next annual report.

Significance to the program of the Cancer Institute: This work may lead to a better understanding of the mechanism of hormone secretion and, hence, radioisotope release in the thyroid gland.

Proposed course of project: To be continued.

Part B included No

Serial No. NCI-929
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Vector Analysis of Ultraviolet Spectra of Ribonucleic Acid Hydrolysates

Principal Investigators: James C. Reid and Arnold W. Pratt

Man Years (calendar year 1960):

Total:	1-1/3
Professional:	2/3
Other:	2/3

Project Description:

Objectives: This project was initiated to study the feasibility of analyzing multi-component mixtures by means of their ultraviolet mixture spectra. Ultimately it is intended to employ the technique to the analysis of urinary excretion patterns in leukemia (see project NCI-919). Before this can be done it is necessary to work out a number of technical problems. To study these, ribonucleic acid is used as a model. The use of ribonucleic acid hydrolysates provides a convenient source of mixtures whose components are well characterized and in addition offers the possibility of developing as a byproduct a method of general biochemical utility for the expeditious determination of nucleic acid base ratios.

Methods Employed: Nucleic acid hydrolysates are analyzed by established chromatographic techniques to provide a nominal standard of reference. Values so obtained are compared with values computed from a large number of observations made upon the ultraviolet spectra of the mixtures. The computations are accomplished by the techniques of vector analysis with the aid of a digital computer.

Major Findings: It has been found that a ribonucleic acid hydrolysate consisting of the four major nucleotides plus three extra components present in smaller amounts can be analyzed by the computational method with a precision equal to or better than that of standard methods and accuracy about as good. In the

trials conducted so far, the spectra of the minor components were not available. It is anticipated that when these have been determined the accuracy will improve.

Work so far has been accomplished by manual methods of data reduction. To eliminate this handicap an automatic system is being assembled. A major component of this system is an analogue-to-digital converter which has been constructed by Mr. Coffey of this Laboratory for attachment to the recording spectrophotometer. This unit is currently under test.

Significance to the program of the Cancer Institute: The techniques developed in this project will be applied to the analysis of urinary excretion patterns currently being obtained on leukemic patients.

Proposed course of project: Completion of the system for reducing data to digital form on paper tape entails receipt of a Flexowriter, scheduled for delivery in the Spring. Pending its arrival additional computer programs necessary for the next phase of the work are being devised (Dr. Pratt). When these are in being it will be possible to determine more adequately the necessary library of individual vectors (spectra) as well as investigate sources of error. Further investigation of the RNA analysis will be conducted to improve its accuracy and the technique will be extended to more complex systems to define the limits of its applicability.

Part B included Yes

PHS-NIH
Individual Project Report
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Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Reid, J. C., and Pratt, A. W.: Vector analysis of ultraviolet mixture spectra: The composition of ribonucleic acid. Biochem. Biophys. Research Communications 3: 337-342, 1960.

Serial No. NCI-931
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Water Exchange in Tumors

Principal Investigators: Florence K. Millar and Julius White

Cooperating Units: Surgery Branch, NCI, Dr. Jack H. Bloch, Serial No. NCI-10-762 (c), Part 4

Man Years (calendar year 1960):

Total:	3-1/3
Professional:	1
Other:	2-1/3

Project Description:

Objectives: To compare the rate of equilibration of water in a variety of tumors with water in blood and other tissues, with the ultimate goal of determining whether anti-tumor agents affect this equilibration and in what direction. It is hoped that this study may shed some light on the feasibility of localizing anti-cancer agents in tumors.

Methods Employed: Tritiated water in tracer amount was injected intravenously into rats and mice bearing any one of a variety of transplanted or primary tumors. The rat tumors were: the Walker 256 carcinosarcoma and Novikoff hepatoma transplanted subcutaneously; the Murphy-Sturm lymphosarcoma transplanted intramuscularly; a primary fibrosarcoma induced by subcutaneous injection of methylcholanthrene; a primary hepatoma induced by butter-yellow feeding. The mouse tumors were the C_3HBA adenocarcinoma transplanted subcutaneously and the hepatoma 134 (an ascites tumor) inoculated intraperitoneally. The animals were maintained continuously under ether anesthesia.

At varying times after tritium injection, animals were sacrificed and samples of cardiac blood, viable tumor, rectus muscle, liver and skin removed. In some cases samples of necrotic tumor tissue were also taken. Water was vacuum-distilled from the tissue and blood samples, and aliquots

of the distillate were measured for tritium content by liquid scintillation radio-assay. The degree of equilibration of a tissue was calculated as the ratio of tritium specific activity of the water in that tissue to specific activity of the water in the respective cardiac blood sample.

Major Findings: With the exception of the mouse ascites tumor, all viable tumor tissues reached complete equilibrium with blood in 5-10 minutes under these conditions of ether anesthesia. In the rat bearing the Walker tumor, this rate of equilibration of tumor was considerably slower than that of liver, which equilibrated in less than a minute. Tumor equilibration rate was similar to that of muscle and somewhat faster than that of skin. The water of necrotic tissue of the Walker tumor equilibrated more slowly than did viable tumor water, but the necrotic center of this tumor did eventually reach equilibrium with the blood in 3-24 hours. In contrast to the viable tissue of the other tumors, the mouse ascites tumor required 30 minutes to reach equilibrium, and the rise in specific activity relative to blood was almost linear with time.

The results indicated that the major factor in regulating water exchange in tumor tissue was blood supply to the tumor.

Significance to the program of the Cancer Institute: The exchange of water between blood and tumor tissue provides indirect evidence of the rates at which other molecules, e.g. chemotherapeutic agents, might be expected to equilibrate if they behaved in a manner analogous to tritiated water.

Proposed course of project: To study a few anti-cancer drugs and determine their rates of equilibration.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Bloch, J. H., Millar, F. K., Harris, A. R., Berlin, N. I., and White, J.: Water exchange in animal and human tumors. (Accepted for publication; to appear in J. Appl. Physiol., Jan. 1961.)

Serial No. NCI-911
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Total Metabolism

Principal Investigators: A. W. Pratt and W. C. White

Man Years (calendar year 1960):
Total: 2
Professional: 1-2/3
Other: 1/3

Objectives: The study of the total metabolism and energy metabolism of tumor-bearing animals. Particularly to evaluate the tumor-bearer by direct and indirect calorimetry.

Methods Employed: The studies utilize, in addition to routine chemical and physical determinations, the techniques of analytical respirometry as developed in this laboratory (Serial No. NCI-911, 1957, 1958, and 1959).

Major Findings: Instrumentation designed to increase the accuracy and reliability of the various transducers used in total energy metabolism measurement by direct and indirect calorimetry has continued to involve a considerable fraction of the total effort in this study. A new carbon dioxide analyzer was designed and constructed using thermistor elements as the heat conduction sensors in a thermal conductivity bridge circuit. In this application it is necessary that the thermistor pair used have matched dynamic as well as matched static characteristics. Matched pairs as specified were obtained and the working analyzer cell appears to have a reasonable stability and about twice the sensitivity of the old hot wire cell. The design of the direct calorimeter has been improved and a better balance and increased sensitivity of the temperature control has been achieved. The temperature of the effluent air is now measured to monitor and record the small amount of heat removed from the calorimeter by the air flow. A wide-range humidity element has been installed which appears to give a good measure of the water vapor in the air leaving the calorimetric chamber. With the addition of new transducers it was necessary to rewrite the computational program. The new IBM-650 program was written to include the additional measurements and was improved with respect to handling the data and errors in the data and also to include additional useful calculations.

An investigation of the use of density measurements for the calculation of gross body composition of the laboratory animal has been initiated and considerable progress has been made with the analysis of dry total carcass composition. A number of animals were chemically separated into dry and non-fat fractions and the specific gravities determined with a 50 ml. pyknometer using kerosene as the fluid for the non-fat fraction and distilled water for the fat fraction. The average densities on this limited number were 1.426 for the non-fat and 0.923 for the fat fraction. If x is the non-fat fraction and y is the fat fraction:

$$\frac{x}{1.426} + \frac{y}{0.923} = \frac{1}{D_{tc}} \qquad (1)$$

where D_{tc} is the density of the dry total carcass.

By using the density measurement of the dry total carcass combined with the bomb calorimetric measurement of the calories per gram of dry total carcass (C_{tc}) and the previously established relationship,

$$4.65x + 9.28y = C_{tc} \qquad (2)$$

one is able to solve for the non-fat and fat fractions without the necessity of chemical separation.

Significance to the program of the Cancer Institute: Total metabolism studies offer the single precise measure of an animal's general metabolic response to tumor growth.

Proposed Course of Project: To continue, but emphasizing the simultaneous measurements of energy loss and energy production in the tumor-bearing animal.

The simultaneous measure of energy expenditure by both direct and indirect means is a most difficult measure because of the precision required in all measuring-recording components. It requires continuous measure over periods of days. Failure in any of the multiple transducers, even if temporary, prevents the precise comparison of direct and indirect calorimetric data. The obvious value of knowing that the independent measures of energy expenditure of the tumor-bearing animal agree or disagree cannot be overstressed. It is impossible to derive the mathematical representation of total metabolism without this knowledge. Since the measurements are only as good as the instrumentation capabilities, the methods thus remain our primary concern.

In addition, a study of the measurement of total-body water of the animal in the metabolic chamber has been initiated in collaboration with Dr. F. K. Millar. By collecting the respiratory water after injecting the animal with tritium-labeled water it is possible to obtain samples at any desired interval without disturbing the animal. The mathematical approach to the solution of the dynamic water pool determination as well as the mathematics of the determination of the body volume of an animal in the respiratory chamber has been formulated. Preliminary experiments show that the measure of total-body water and of body volume of the animal in the calorimeter is possible but will require very accurate measurements and precise experimental procedures to attain significant results. However, if the necessary precision is achieved it will then be possible to reduce the density and caloric measurements done on the living animal in the calorimeter to a dry body carcass basis and solve for the non-fat and fat fractions in the intact animal.

Part B included No

Serial No. NCI-911(a)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Physiological Regulation of Human Body Temperature

Principal Investigators: A. W. Pratt, H. Benzinger* and C. Kitzinger*

Cooperating Unit: *Bioenergetics Division, Naval Medical Research Institute

Man Years (calendar year 1960):
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objectives: The Energy Metabolism Section of the Laboratory of Physiology is attempting to measure the energy metabolism of tumor-bearing subjects. Much of our work has been devoted to the development of analytical respirometry techniques for such studies. The intimate relation between energy metabolism and body temperature is obvious. We are hoping that the cooperative studies at Naval Medical Research Institute on normal human subjects will allow development of the techniques of clinical thermometry and analytical respirometry which will afford new approaches to the study of total metabolism in the human subject.

Methods Employed: Direct calorimetry (NMRI instrument)
Analytical Respirometry (NCI instrument)
Thermocouple thermometry

Major Findings: The human's response to cold ambient atmospheres by increased heat production was found to be quantitatively related to skin temperature and is also dependent on cranial internal temperature in a meaningful, quantitative and reproducible manner. A plot of an individual's observed heat production (c.c. O_2 consumed per min.) against a range of skin temperatures gives for

each intracranial temperature (measured in the nasopharynx or on the tympanic membrane) a smooth curve with a minimum at approximately 16° C, going through a maximum at approximately 23° C and reaching a minimum at approximately 29° C of measured skin temperature. This is a basic relationship between skin temperature, intracranial temperature and energy production. The use the clinic can make of this generalized observation must await further clarification. Particularly important will be a knowledge of the thermal threshold values for the central nervous system components which regulate body temperature.

Significance to the program of the Cancer Institute: Knowledge of the basic, quantitative functions of human temperature regulation is important unto itself. The use of precise clinical thermometry techniques coupled with metabolic studies is the desired and needed application.

Proposed course of project: To continue the cooperative studies.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1960

Part B Honors, Awards, and Publications

Publications other than abstracts from this project:

Van Liew, H. D., and Pratt, A. W.: A gravimetric method using expired air for preparing precise calibration mixtures for a respiratory gas analysis instrument. Naval Medical Research Institute, Bethesda, Md., MR 005.14 - 3001.05 Report No. 1, pp. 287-293 of Institute Report Series of 1960.

Serial No. NCI-911(b)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Mass Spectroscopy

Principal Investigator: B. E. Burr

Cooperating Units: NCI, Radiation Branch -
Dr. Charles R. Maxwell and Dr. Peter Riesz
(Serial Nos. NCI-697 and 629)

Man Years (calendar year 1960):
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To provide both analytical and isotope ratio mass spectroscopy.

Methods Employed: Analytical mass spectroscopy.
Isotope ratio mass spectroscopy.

Major Findings: Both the isotope ratio and the analytical mass spectrometry have continued to be handled by Mr. Burr. Much of the routine work in this area contributes directly to the activities of this Laboratory. (Standardization of reference gas mixtures, isotope ratio determinations, etc.) These measurements provide essential references for our physiological investigations. These mass spectrometry facilities have been available and have been utilized, on a service basis, by various other N. I. H. investigators for special problems (J. Hickey, R-OC, Analysis of gas in sterile feed pockets; Dr. A. Weissler, H-LTD, Analysis of argon-di-ethyl ether mixtures; Dr. M. B. Lipsett, C-E, Studies of the effects of endocrines upon dwarfism in humans; Dr. D. P. Tschudy, C-GM, Studies of size and rate of turnover of nitrogen metabolic pool and rate of body protein synthesis in humans, and Effects of irradiation and tumor growth on these variables.) Additionally, Mr. Burr is continuing the collaboration with members of the Radiation Branch in studies of the

Serial No. NCI-911(b)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Mass Spectroscopy

Principal Investigator: B. E. Burr

Cooperating Units: NCI, Radiation Branch -
Dr. Charles R. Maxwell and Dr. Peter Riesz
(Serial Nos. NCI-697 and 629)

Man Years (calendar year 1960):
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To provide both analytical and isotope ratio mass spectroscopy.

Methods Employed: Analytical mass spectroscopy.
Isotope ratio mass spectroscopy.

Major Findings: Both the isotope ratio and the analytical mass spectrometry have continued to be handled by Mr. Burr. Much of the routine work in this area contributes directly to the activities of this Laboratory. (Standardization of reference gas mixtures, isotope ratio determinations, etc.) These measurements provide essential references for our physiological investigations. These mass spectrometry facilities have been available and have been utilized, on a service basis, by various other N. I. H. investigators for special problems (J. Hickey, R-OC, Analysis of gas in sterile feed pockets; Dr. A. Weissler, H-LTD, Analysis of argon-di-ethyl ether mixtures; Dr. M. B. Lipsett, C-E, Studies of the effects of endocrines upon dwarfism in humans; Dr. D. P. Tschudy, C-GM, Studies of size and rate of turnover of nitrogen metabolic pool and rate of body protein synthesis in humans, and Effects of irradiation and tumor growth on these variables.) Additionally, Mr. Burr is continuing the collaboration with members of the Radiation Branch in studies of the

abstraction reactions of hydrogen atoms in radiochemical reactions of organic compounds in aqueous solutions (NCI-697); also in studies of the irradiation of amino acids (NCI-629).

Significance to the program of the Cancer Institute: To afford this unique measurement to interested scientists.

Proposed Course of Project: Our program requirements for precise analytical mass spectroscopy are small. There is some demand by other scientists for this service as evidenced by the report above.

This service function has been possible because of Mr. Burr's availability. He is now contemplating a change of job. It is proposed that the Laboratory of Physiology discontinue any analytical mass spectroscopy work.

Part B included No

Serial No. NCI-914
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: In Vivo Measurement of Tumor pH with the Capillary Glass Electrode

Principal Investigator: H. Kahler

Other Investigators: B. Moore

Man Years (calendar year 1960)
Total: 2
Professional: 1/3
Other: 1-2/3

Project Description:

Objectives: The object of this project is to determine the in vivo pH of tumors under different experimental conditions.

Methods Employed: Fine-pointed glass electrodes, selected after passing tests for reliability, were inserted into tumors in the leg muscle of rats and the pH recorded for several hours. At the end of the first hour glucose was injected intravenously or intraperitoneally and the effect of this upon the pH was observed. At one-half to one hour before the end of the experiment a solution of Lissamine green dye (Goldacre and Sylvén) was injected into the rats intravenously or intraperitoneally. After chloroforming the animals the tumors were dissected noting the positions of the glass electrode channels.

Major Findings: The green dye in the blood vessels stains the tumor green if the tumor has a good blood supply. If the blood circulation is poor the tissue remains white, if there are pockets of blood in a static condition, it remains red. It was found that all of the pH-time curves which showed a rapid drop and recovery of pH with time were obtained from those glass electrodes which had been inserted into tissue with a good blood circulation as indicated by a green coloration of the tissue.

Significance to the program of the Cancer Institute: The pH of tumor tissue is essential to an understanding of chemical processes in the tumor.

Proposed Course of Project: This program has been concluded.

Part B included No

Serial No. NCI-915
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Cellular Particulates

Principal Investigator: H. Kahler

Other Investigator: B. J. Lloyd, Jr.

Cooperating Units: NCI, Laboratory of Biology, Leukemia Studies Section - Dr. L. W. Law (Serial No. NCI-427).
NCI, Laboratory of Pathology - Dr. C. J. Dawe (Serial No. NCI-521a).
NIAID, Laboratory of Infectious Diseases - Dr. W. P. Rowe (Project Serial No. not available).

Man Years (calendar year 1960)
Total: 2
Professional: 2/3
Other: 1-1/3

Project Description:

Objectives: Parotid Tumor Agent or Polyoma Virus -- The relation between concentration of particles as determined by electron microscope technics and various biological effects.

Methods Employed: Partially purified virus of four polyoma strains was sedimented onto collodion films mounted in ultracentrifuge cells. The concentration of the virus was then determined by counting electron microscopic images of the virus. Using this information the four strains were brought to similar concentrations of virus by dilution. These solutions were then injected into newborn mice for tumor induction assays. Dilutions of 10^2, 10^4 and 10^6 were also injected. Aliquots were used for cytopathogenic effect, HA and HI titer.

Major Findings: The different strains have different biological responses. The most remarkable of these is the P388 strain of Dawe. In the undiluted and 10^2 dilutions thymic epithelial tumors develop while in the 10^4 and 10^6 dilutions unilateral parotid tumors develop.

The non-oncogenic strain of Rowe (397) on the other hand develops parotid tumors only in the undiluted extract.

The number of particles to induce tumors varies from 20 to 1,000,000 depending on the virus strain.

Polyoma Virus Filtration

It was observed that millepore filters of pore diameter 450 millimicrons, 10 X the diameter of the polyoma virus, would not pass the virus in aqueous buffer solutions. The traditional way of filtration is to add beef broth to the virus extract. It was found that bovine albumin, as well as beef broth, would enable the virus to pass through the filters. With high dilutions of albumin the virus is passed in only small amounts but a suspension of virus made up with albumin 1/2 percent readily passes these filters.

Significance to the program of the Cancer Institute: Provides evidence bearing on the virus concept of some cancers.

Proposed Course of Project: This program has been discontinued due to the retirement of Dr. H. Kahler.

Part B included No

Serial No. NCI-916
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1960

Part A

Project Title: Characterization and Comparison of Nucleic Acids, Nucleoproteins, Nucleases, and Nuclease Inhibitors of Normal and Malignant Tissues and of Body Fluids

Principal Investigator: Joseph Shack

Man Years (calendar year 1960):
Total: 2-1/3
Professional: 1
Other: 1-1/3

Project Description:

Objectives:

a) Characterization of the nucleic acids and nucleoproteins of normal and malignant tissues and of tumor viruses in terms of their physical, chemical and biological properties.

b) Development of new procedures for study and characterization of nucleic acids and nucleoproteins.

c) Determination of the nature and amount of nucleases and nuclease inhibitors present in tissues and body fluids; elucidation of factors governing the action of these enzymes; determination of the mechanism of action of these enzymes on nucleic acids and of the nature of the products of such action.

Methods Employed:

a) Procedures for isolation of proteins, nucleic acids, and nucleoproteins in a native condition, and for controlled modifications of these by such agents as acid, alkali, heat, specific enzymes, various chemicals; study of the original and modified materials by various physical, chemical, and, when possible, biological procedures.

Major Findings: Considerable attention has been given to a study of the action of the neutral deoxyribonuclease (DNase I) on deoxyribonucleic acid (DNA) over a wide range of conditions in order to clarify several questions which have remained obscure for many years. The results have shown that the various conditions that affect activity (as pH, substrate concentration, type and concentration of cations and of non-specific salts) are not independent but rather interdependent variables. For example, the requirements for Mg^{++} vary with the pH and with the substrate concentration, and the dependence of the Mg^{++} requirement on substrate concentration is quite different at different pH values. Likewise the pH activity curves and the apparent pH optimum vary with both Mg^{++} and DNA concentration. Contrary to many earlier reports it was found that proper control of these variables yields a substrate concentration-activity relation which corresponds to classical Michaelis-Menten kinetics.

A detailed study was made of the so-called synergistic effect of Ca^{++} in raising the DNase activity in the presence of Mg^{++}. Under certain conditions of pH and substrate concentration, higher activities can be attained by combinations of Ca^{++} and Mg^{++} than by Mg^{++} alone. However, the highest activity reached by the best combination of Ca^{++} and Mg^{++} never exceeds the maximum activity obtained with Mg^{++} alone at optimal pH and relatively high DNA concentration. It was found that the activating effect of Ca^{++} occurs only when the enzyme activity is reduced below this maximum by a lowering of DNA, a rise of pH, a rise of salt concentration or by combinations of these. The effect of Ca^{++} appears to result from a lowering of the Michaelis-Menten constant below that characteristic of the Mg^{++} activated systems. Analysis of these effects suggests that two cations are concerned in forming the complex between one DNase molecule and DNA. These may be two magnesium ions, two manganese ions, or one magnesium and one calcium ion.

It was found that the rate of hydrolysis of DNA falls off rapidly to a very low value when less than half of the susceptible bonds are broken. Detailed analysis of this effect shows that, contrary to previous reports, it is neither due to inhibition of the enzyme by products of the reaction, nor to reduction of substrate. Instead it appears that DNA contains linkages of widely different susceptibility to the enzyme, and that the rate falls off as the more susceptible linkages are broken. Comparison of results with native and denatured DNA indicates that the differences of susceptibility are not dependent on the specific secondary structure possessed by native but not by denatured DNA, but are more probably related to the sequence of nucleotides in the vicinity of the particular bond.

Significance to the program of the Cancer Institute: The significance of research on nucleic acids and nucleoproteins in connection with the cancer problem is indicated by the key role of these substances in genetic processes, bacterial and viral transformations, virus reproduction, protein synthesis. Interest in nuclease and nuclease inhibitors relates to the necessity of controlling DNase action during isolation of DNA, particularly if it is to be tested for biological activity. It is also possible that the amount and nature of the enzymes and enzyme inhibitors may be in some way related to the growth of tumors present in the host. Finally, the nucleases serve as useful and important tools in studies of both the primary and secondary structure of nucleic acids.

Proposed Course of Project: In addition to continuation along the lines covered in this report it is contemplated that increasing attention will be given to related studies of the more homogenous nucleic acids that can be isolated from viruses, including the tumor viruses. It is hoped that such work may develop useful knowledge regarding correlation of the structure and biological activity of nucleic acids.

Part B included No

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